Development of Liquid Atmospheric Pressure MALDI for Microbial Profiling and Clinical Biotyping

A Thesis Submitted to the University of Reading in Partial Fulfilment for the Degree of Doctor of Philosophy

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November 2023

Abstract

Clinical mass spectrometry (MS) has had great success in the last two decades for the identification of microorganisms using matrix-assisted laser desorption/ionisation (MALDI) MS biotyping. In this thesis, the application of the more recently developed liquid atmospheric pressure (LAP) MALDI has been applied to the analysis of clinically important samples for biotyping and diagnostic purposes. LAP-MALDI MS allows the detection of ESI-like protein ion signals, whilst maintaining the benefits of traditional MALDI such as high tolerance to contamination and providing additional advantages such as stable and homogenous sample droplets and in turn, stable ion flux.

Firstly, the exploration of MS profiling will be discussed, with the identification of clinically relevant bacteria performed via unsupervised statistical analysis of unique lipidomic profiles. This also encompasses the first LAP-MALDI mass spectra of bacteria possessing lipids and proteins in the same spectrum. Following on from this, antimicrobial resistance was investigated via the multiplex detection of antibiotic hydrolysis in the presence of bacteria possessing β-lactamase resistance genes. In this assay, the lipidomic profiles for bacteria were still present, therefore AMR detection and species identification can be coupled in a single assay.

Finally, LAP-MALDI's potential use in infectious disease detection was also explored in animals. Bovine tuberculosis poses a high risk to cattle health and UK farming, and therefore efficient diagnostics are vital. The use of LAP-MALDI MS allows the detection of a key inflammatory protein (S100-A12), which is correlated to infection in cattle. Linear discriminant analysis (LDA) shows the discrimination between healthy cattle, and two disease states with high sensitivity and specificity. Collectively, this thesis highlights the

valuable contribution LAP-MALDI could present in clinical diagnostics, providing more rapid and more informative diagnostic results, for both humans and animals.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Sophie Lellman

Acknowledgements

Firstly, I would like to thank Professor Rainer Cramer for all his advice and support throughput my PhD. He has provided me with many valuable opportunities, which I am extremely grateful to have been involved in. He has always provided invaluable feedback and motivation. I also thank members of the Cramer group, both past and present. In particular Lily Adair, there is no one else I would rather have to take this project further than I have started.

To Nisha Patel, my amazing manager at UCB, who always reassures me of my abilities. I cannot thank her enough for being there for me since I started, as a manager and a friend.

Thank you to my friends and family for all the support they have given me; from the laughs to a shoulder to cry on, and everything in between.

Finally, the biggest thank you of all goes to my Mum, who is my biggest cheerleader. She has been there for every high and low of the last five years, any second of the day that I have needed her. She's encouraged me never to give up, even when I wanted to. I would never have been able to finish it without her.

I would like to dedicate this work to my Dad.

Contents

Abbreviations

ABC – ammonium bicarbonate

ACN - acetonitrile

AMR – antimicrobial resistance

APHA – animal and plant health agency

AP – atmospheric pressure

AST – antimicrobial susceptibility testing

bTB – bovine tuberculosis

Da - Dalton

DC – direct current

DEFRA – department for environment, food, and rural affairs

DHB – dihydroxybenzoic acid

DTT - dithiothreitol

CHCA – α-cyano-4-hydroxycinnamic acid

CID – collision induced dissociation

ECD – electron capture dissociation

EG – ethylene glycol

ESI – electrospray ionisation

ETD – electron transfer dissociation

EtOH – ethanol

FA – formic acid

H_2O – water

HPLC – high-performance liquid chromatography

HT – high-throughput

Hz - hertz

IAA – iodoacetic acid

IgA – immunoglobulin A

IFN-ɣ - interferon gamma

kDa - kilodalton

kV - kilovolt

LAP-MALDI – liquid atmospheric pressure matrix-assisted laser/desorption ionisation

LDA – linear discriminant analysis

L/h – litres per hour

LSM – liquid support matrix

MALDI – matrix assisted laser desorption ionisation

MDRO – multi-drug resistant organism

MeOH – methanol

µJ – microjoule

µL - microlitre

mM – millimolar

MRSA – methicillin resistant *Staphylococcus aureus*

MS – mass spectrometry

MS/MS – tandem mass spectrometry

NCTC – national collection of type cultures

nm – nanometre

ns - nanosecond

PCA – principal component analysis

PCR – polymerase chain reaction

PBS – phosphate buffered saline

PG – propylene glycol

ppm – parts per million

REIMS - rapid evaporative ionisation mass spectrometry

rpm – revolutions per minute

RT-PCR – reverse transcription polymerase chain reaction

SICCT - single intradermal comparative cervical tuberculin test

SOP – standard operating procedure

spp - species

TCA – trichloroacetic acid

TFA – trifluoroacetic acid

TOF – time of flight

QTOF – quadrupole time of flight

UTI – urinary tract infection

UV – ultraviolet

V - volt

Related Publications

The publications listed here are supporting this thesis but are not directly related to the main publications forming chapters 3-7.

Title – Multi-omic MS(/MS) analysis and identification of bacteria using liquid atmospheric pressure (AP) MALDI

Citation – Lellman, S. E., and Cramer, R. In: ASMS 2020 Reboot, June 1-12, Online Poster.

Abstract - Biotyping using mass spectrometry is commonly used in the clinical laboratory, which has transformed microbial identification in hospitals. Here, we present the application of liquid AP-MALDI MS to the analysis of clinically relevant bacteria.
Liquid AP-MALDI is an emerging MALDI technique, arguably providing many benefits over traditional MALDI, performed *in vacuo* with solid samples. The use of a self-healing liquid droplet for analysis provides a homogeneous distribution of matrix and analyte molecules, in turn resulting in an extremely stable ion flux. An AP ion source also removes the need to reach vacuum conditions and combined with a liquid sample droplet which does not require drying time it offers an overall faster MALDI sample preparation and introduction into the MS instrument.
First-generation bacterial strains were grown according to manufacturer guidelines. Following incubation, bacteria were harvested from solid media and subjected to an ethanol and formic acid inactivation/extraction protocol. A liquid support matrix (LSM) formed of ethylene glycol (70%) and α-cyano-4-hydroxycinnamic acid (25mg/mL) was used for all analyses. LSM was spotted onto a stainless-steel target plate, followed by the addition of analyte solution (1:1). An in-house developed AP-MALDI source with 3kV applied between

target plate and heated capillary inlet tube was coupled to a Synapt G2-Si. Standard operating settings for the source were 3.5kV with 180 L/h N_2 counter-flow gas. Data were acquired with a pulsed nitrogen laser (337nm, 3ns, 10Hz) and processed using MassLynx 4.1® and AMX® (Waters) software.

Analysis of clinically relevant, diverse bacteria shows that the use of liquid AP-MALDI MS allows discrimination and identification of bacteria to the species level based upon their lipid profile. Unique lipid profiles can be found in the m/z region 400-1,100 and allow discrimination between even closely related species such as *Klebsiella pneumoniae* and *Escherichia coli*. Principle component analysis was performed using MS profiles, whereby each species formed distinct clusters, providing a classification accuracy of 98.63%. Ion signals for lipids are typically excluded from commercial biotyping instruments, due to the formation of abundant matrix ions in the lower m/z range (below 2,000).

The liquid AP-MALDI source also allows acquisition of multiply charged ions, such as peptides and proteins. Multiply charged peptides/proteins obtained directly from the same sample used for MALDI biotyping by profiling, greatly improves bacterial identification. In particular, multiply charged peptides/proteins analysed by MS/MS on a high-performing hybrid mass analyzer such as a Q-TOF or an orbitrap instrument provides superior sequencing and structural analysis compared to axial TOF MS/MS.

In combination with the MS profiles of other biomolecules such as lipids, this new liquid AP-MALDI approach provides both extended profiling capabilities and superior biomolecular identification via MS/MS, all extremely rapidly and from the same sample.

First MS/MS data from bacterial biomarkers contributing to species identification and thus improving identification confidence will be presented.

Bacterial analysis using liquid AP-MALDI on hybrid ESI mass analyzers retains (in some cases even improves) the merits of conventional solid MALDI such as speed, off-line and ease of sample preparation, and high tolerance to contaminants, and adds to this all the benefits that ESI analysis is known for. The use of multi-omic spectral profiling and superior structural analysis extends the peptide/protein mass fingerprinting approach, which is currently the only but widely utilized form of identification.

Novel aspect - Species-level bacterial identification using MALDI-MS profile and MS/MS analysis of lipids and multiply-charged peptides/proteins, all from the same sample.

Title – Identification of Bacteria by Proteomic Profiling and Sequencing using Liquid AP-MALDI MS/MS

Citation – Lellman, S. E., and Cramer, R. In: Mass Spectrometry and Advances in the Clinical Laboratory EU Virtual, September 13-17 2021, Online Poster.

Abstract - Clinical diagnosis of bacteria by mass spectrometry (MS) is typically based upon the MALDI MS profile of ribosomal proteins which are matched against a reference database. Closely related species can possess a high degree of similarity in their proteomes, therefore some proteins may be of the same, or very similar, m/z value leading to potential misidentification. Liquid AP-MALDI MS is an emerging technique which has recently been demonstrated for bacterial identification based upon lipid profiles. Here we build upon this identification with the acquisition, and subsequent sequencing, of multiply charged proteins, which, in combination with lipid profiles, has the potential to provide a higher confidence identification than traditional MALDI MS. The objective of this study is to identify different bacterial species based upon MS profile and sequencing of biomarkers using liquid AP-MALDI MS/MS.

Bacteria were grown according to growth guidelines from NCTC. Following the recommended growth period, half a loopfull of biological material was harvested and resuspended in 1mL 1X PBS. Suspensions were prepared using a simple TCA precipitation, with a preparation time of approximately 40 min. C18 ZipTips were used for sample clean-up. An α-Cyano-4-hydroxycinnamic acid based liquid support matrix (LSM) was used for all analyses. 0.5µL of LSM was spotted on the target plate, followed by 0.5µL of analyte. An in-house built AP-MALDI source coupled to a Synapt G2-Si was used at standard operating settings of 3.0kV with 180L/h counterflow N_2 gas for all analyses. A pulsed N_2 laser was

operated at 21µJ/pulse at a repetition rate of 30Hz. MassLynx 4.2® software was used for data acquisition and processing. AMX® software was used for statistical analysis.

Liquid AP-MALDI mass spectra were acquired in the m/z range 100-2,000, with each bacterium possessing a unique spectral profile formed of lipids and/or proteins. Not only does the MS profile contain a higher variety of biomarkers than traditional MALDI mass spectra, the use of an AP-MALDI source allows coupling to high performed hybrid QTOF instruments with MS/MS capabilities. This means that the multiply charged protein signals can be easily fragmentation using CID, subsequently allowing for sequencing of biomolecules. Various proteins were identified from each bacterium, up to approximately 20kDa in molecular weight, including DNA binding proteins, unique to each species. Acquisition of an MS profile allows a rapid identification in 1 minute of analysis time. In cases where this is not sufficient, such as closely related species, MS/MS sequencing can be done to provide a higher confidence identification in approximately 2 min.

Conclusion – Liquid AP-MALDI can be used for bacterial identification using lipidomic and proteomic profiling of bacteria. Additional MS/MS analysis can be performed to increase the confidence of the identification.

Title - Profiling and sequencing of multiply charged proteins for bacterial identification using liquid AP-MALDI MS/MS

Citation - Lellman, S. E., and Cramer, R. In: 41st BMSS Annual Meeting 2021, September 8-9, 2021, Sheffield, UK. Flash poster talk.

Abstract - Liquid atmospheric pressure (LAP)-MALDI MS has recently been applied for the identification of clinically relevant bacteria based upon lipid profiles. Using an altered preparation method, here we present the use of LAP-MALDI MS/MS for bacterial identification based upon ion signals from multiply charged proteins, and their subsequent sequencing by MS/MS. Identification of bacteria via the detection of sequenced peptides and proteins provide a superior accuracy, and thus higher diagnostic confidence, than using protein profiles alone.

Clinically relevant bacteria were grown for the appropriate incubation period. Approximately half a loop full of biological material was harvested and resuspended in 1 mL of 1X PBS. Bacterial suspensions were sonicated in an ultrasonic water bath for 15 min to lyse the bacterial cells. Following sonication, 50 µL of 100% TCA was added to each of the suspensions, which were then left to precipitate on ice for 15 min. Suspensions were subsequently centrifuged at 13,000 rpm for 5 min and the supernatants were discarded. The resultant pellets were resuspended in 0.1% TFA. Samples were then centrifuged again at 13,000 rpm for 5 min to remove insoluble material and the supernatants were harvested, cleaned up with C18 ZipTips and used for analysis.

A CHCA-based liquid support matrix (LSM) was used, formed of 25mg/mL CHCA in 70:30 ACN:H_2O with 70% ethylene glycol. Samples were spotted 1:1 with the LSM and analysed via an in-house developed LAP-MALDI source coupled to a Synapt G2-Si Q-TOF instrument

(Waters). Data was acquired and processed using MassLynx 4.2® software (Waters). Data shows that all clinically relevant bacteria analysed in this study can be separated based on their LAP-MALDI MS protein profile within the m/z range of 100-2000. Principle component analysis and cross validation were performed using AMX Model Builder® (Waters) in order to determine the classification accuracy. MS/MS analysis of multiply charged ion signals within the profile allowed sequencing of proteins, such as DNA binding proteins, unique to each bacterium.

The current gold standard of MALDI MS for clinical biotyping is based upon protein TOF MS profiles in the m/z range of 2,000-12,000. LAP-MALDI MS has the potential to advance this, via the acquisition of highly charged protein signals below m/z 2,000, which is not possible in conventional solid MALDI-TOF MS. Use of an AP MALDI source enables coupling to high performance MS/MS instruments such as the Synapt G2-Si used here, which allows effective MS/MS sequencing of large peptides and proteins, providing highly species-specific information, with little analysis time required.

Title - Clinical and Veterinary Applications of LAP-MALDI MS

Citation - Lellman, S. E. and Cramer, R. In: BSPR Annual Meeting 2022, July 3-6, 2022, Oxford, UK

Abstract - In clinical and veterinary settings, there is always a pressing need for efficient workflows for accurate diagnosis. Liquid atmospheric pressure MALDI MS (LAP-MALDI MS) was first demonstrated for diagnostics for bovine mastitis, and since then many further developments have been made. Following from the successful detection of pre-clinical mastitis based upon lipid, peptide and protein signals, investigations have also turned towards human diagnostics. LAP-MALDI MS offers benefits over traditional MALDI, including the detection of multiply charged ions and subsequent MS/MS, high-throughput analysis and low interference from contaminants and matrix ion peaks.

Sample preparation techniques were optimised depending on the biological sample. For bacterial profiling, both EtOH:FA extraction and TCA precipitations were used for the detection of lipids and proteins respectively. A simple, rapid incubation assay following by EtOH extraction was used for assessment of antibiotic resistance in bacteria. For bovine samples, a short digestion step can be included to assist in the detection of biomarkers for bTB diagnostics. All samples were analysed via LAP-MALDI MS using a CHCA based liquid support matrix. A Synapt G2-Si (Waters) with an AP-MALDI source was used for all analyses.

LAP-MALDI MS/MS has the ability to identify human pathogens using lipidomic and proteomic fingerprints, with the additional identifying power of peptide/protein sequencing. This provides definitive confirmation of the identity of biomarkers within a sample. Furthermore, LAP-MALDI MS has been demonstrated for the detection of antibiotic resistant bacterial strains. This has been developed as a rapid one-pot multiplex assay which

allows assessment of resistance/susceptibility of a panel of antibiotics, together with a unique lipidomic profile within the same spectrum. This allows identification of the pathogen, as well as resistance and/or susceptibility to multiple antibiotics in one rapid assay, which, in a clinical setting, can lead to more rapid diagnosis of infection, allowing more informed decisions on treatment options, ultimately resulting in better patient prognosis. LAP-MALDI has also been recently explored in bTB diagnostics. A large-scale analysis of nasal swab samples from healthy cows, and those with either bTB or bovine mastitis revealed the potential for LAP-MALDI MS to provide a triage system for the detection of disease in cows. This could reduce the need for widespread testing and reduce the impact on the animals, farmers, and veterinary staff.

Chapter 1 - Introduction

Microorganisms can be harmless or even beneficial to the human body. In the gut, bacterial species such as *Bifidobacteria* and *Lactobacillus* are highly advantageous to a healthy microbiome, contributing to good intestinal health.(1) However, there are also many species that can cause disease under certain conditions. Infectious diseases that are caused by microorganisms resulted in 442,869 hospital cases in the UK between 2020 and 2021.(2) Therefore, the identification of micro-organisms is an essential diagnostic tool in the treatment of disease. Traditional methods of identification have been surpassed by newer technologies, allowing more rapid diagnosis, resulting in better patient prognosis. In this context, traditional methods encompass the use of phenotypic assays such as gram staining and biochemical assays.(3) These tests give results that define certain characteristics of the microorganism, such as the composition of the cell wall or sugar fermentation, rather than direct identification of the organism. Traditional assays have formed the basis of microbiological identification, however the lack of specificity of identification, as well as the time taken for the read-out to be performed, means there is a pressing need for newer technologies to overcome these issues. Identification from traditional assays requires an initial culture, which typically has a minimum incubation time of 18 h but can be up to 14 days, depending on the organism.

For instance, catalase tests are used to assist in the identification of Enterobacteriaceae, producing an immediate result upon observation (or lack) of effervescence in the presence of hydrogen peroxide.(4) However, this test can only distinguish down to a genus level. A citrate test is also used for identification of Enterobacteriaceae, to determine the use of citrate as an energy source. With the citrate test, the initial bacterial culture is performed, biological material harvested, and citrate agar is inoculated followed by a second incubation

period.(5) The secondary incubation period can take up to an additional 7 days, and therefore poses a significant time-limiting impact on identification. In patients that have septicaemia, for example, it is imperative a timely diagnosis is provided. Suitable care packages are required for septic patients within 24 h in order to reduce mortality.(6)

Newer technologies have been able to overcome some of the obstacles posed by the traditional assays. Mass spectrometry and molecular assays have transformed microbial identification in clinical labs.(7, 8, 9) Molecular assays are often targeted for the identification of specific microorganisms. Polymerase chain reaction (PCR) assays are used to identify specific DNA sequences that are unique to each species or sub-species.(10) They are highly specific, use little biological material and do not require additional culture time. However, there must be prior knowledge to the potential identification of the microorganism, as specific DNA primers are required that are complementary to the sequence of the microbe. Often a panel of primers are run in order to maximise identification capacity, however the use of primers come at a high cost to the laboratory and can be of limited supply. This was observed in the recent COVID-19 pandemic, which heavily relied on the use of PCR testing for diagnosis of disease.(11) Many drawbacks to the use of RT-PCR as the gold standard for COVID-19 testing have been detailed, including the detection of false positives due to viral shedding following the infectious period, as well as technical sample preparation issues.(12) The widespread use of PCR testing for SARS-CoV-2, however, does indeed highlight its usefulness in microbial identification.

1.1 Clinical Mass Spectrometry for Microbial Analysis

The use of mass spectrometry in clinical laboratories has revolutionised microbial identification, in particular for bacteria, mycobacteria, yeast and funghi. Anhalt and Fenselau first used mass spectrometry for bacterial identification in 1995, detecting

pyrolysis products of phospholipids and ubiquinones to profile gram-negative bacteria.(13) In the present day, MALDI-TOF biotyping instruments are approved for use in many clinical laboratories; identification is performed based upon the analysis of a protein mass fingerprint, unique to each microorganism, which is matched against a reference database.(14) Analysis is typically performed in the m/z region of 2,000-20,000,(15) with the mass fingerprint formed of typically singly charged ions from ribosomal proteins. In the case of the Biotyper (Bruker), results are delivered as a confidence score between 0.0-3.0, allowing simple readout of results rather than in depth analysis of a mass spectrum. There is little sample preparation involved; biological material from a single colony is sufficient, harvested with a toothpick, and transferred to the target plate. Results can also be provided within 5 min per sample, in comparison up to 48 h for conventional methods.(16) A matrix is overlaid, typically α-cyano-4-hydroxycinnamic acid (CHCA), and the sample can be directly analysed. Where this direct transfer method does not produce a sufficient identification, an ethanol and formic acid extraction can be used. Clinical biotyping instruments include the Biotyper (Bruker) and VITEK MS (bioMerieux). Many studies have investigated the efficacies of commercial biotyping against each other as well as alternative methods. With the ASTA MicroIDSys (ASTA) being more recently developed, the majority of the comparative studies involve the Biotyper and VITEK MS. Evidence for both systems being superior over each other have been published.(17, 18) A limiting factor of MALDI MS biotyping instruments is the extent of the databases; there must be a profile in the database to match, in order to provide a result. This means that certain strains, which may be rarer, or possess a mutation leading to a new strain, cannot be identified. There are also certain species that are closely related, which are difficult to discriminate. Enteroinvasive *E. coli* and *Shigella* spp are commonly difficult to discriminate due to their high levels of genetic relatedness, making

diagnosis by any means challenging. MALDI biotypers are limited in the identification of these species,(19) as well as *Enterobacter* (20) and *Neisseria* spp(21).

Ultimately, there are several benefits of MALDI MS biotyping systems. The reduction for time taken for a diagnosis is a key advantage over traditional diagnostics. Identification of bacterial from stool samples can take up to 3 days using selective culture methods; MALDI MS can identify species within 30 min.(22) The rapid identification of bacteria leads to faster initiation of patient treatment, in turn leading to better patient prognosis.(23) Besides the initial outlay of the instrument costs, MALDI biotyping drastically reduces the costs of bacterial identification in comparison to traditional methods. The reagents required for traditional methods can be expensive; these are not required with MALDI MS and can save clinical laboratories up to 50% annually.(24) MALDI MS biotypers also have the ability to detect microorganisms directly from blood culture. This vastly reduces the turnaround time for identification further, as a solid culture is not required for analysis.(25) MALDI MS biotyping has also been applied for veterinary diagnostics following culture; however, most veterinary pathogens that are present in commercial databases are those that also infect humans. Small genetic differences may influence the accuracy of veterinary diagnostics.(26) Biotyping using MALDI MS can also be applied to other microorganisms, such as yeast and fungi. Many commercial databases include clinically relevant pathogens which belong to these groups. Fungi and yeasts are larger in size in comparison to bacteria, and require additional sample preparation for protein extraction, which often includes an ethanol and formic acid extraction protocol.(27)

1.2 Mass Spectrometry; Soft Ionisation Techniques

MALDI is one of the two major types of ionisation techniques used in mass spectrometry, along with electrospray ionisation (ESI). Both MALDI and ESI are described as 'soft'

ionisation techniques, as they are less destructive to analytes, allowing analysis of intact biomolecules. ESI is often coupled to chromatography systems for additional separation of samples with complex biological matrices. Separation of analytes by chromatography are dependent on their interaction with the stationary and mobile phases. This is often useful in the separation of complex bottom-up protein analysis, whereby a digestion is performed resulting in the production of several peptides. Enzymatic digestion is commonly performed in order to assist in the identification of larger proteins, due to the specificity of the enzymes. Trypsin is a highly specific enzyme, cleaving at lysine and arginine residues. Tandem MS, or MS/MS, is easily performed with ESI MS, and greatly assists in definitive identification of proteins. Tandem MS will be discussed in section 1.3.

With MALDI MS, as alluded to above, a matrix compound is used to assist desorption/ionisation of analytes. Matrix compounds are typically selected for optimal ionisation dependent on the wavelength of the laser used, as well as the type of analyte, mode of ionisation, sample purity, too name but a few. The most commonly used matrices are α-cyano-4-hydroxycinnamic acid (CHCA) and 2, 5-dihydroxybenzoic acid (DHB). Both CHCA and DHB absorb strongly at 337nm and 355nm, and as a result lasers of these wavelengths are commonly utilised in MALDI sources. For conventional MALDI sample preparation, the matrix is co-crystallised with the analyte on a target plate, and the laser is directed towards the sample. A common issue of crystalline MALDI samples is the need to manually search for the 'sweet-spot' of the sample to obtain optimal signal intensity for analyte ions. The use of defined or random pathways can attempt to mitigate this issue to ablate maximal material from the sample spot, however will not overcome the ion flux stability issue. Sample preparation is a vital part in MALDI MS. The choice of matrix is often based upon the type of analyte expected. DHB is typically preferred for the analysis of

larger biomolecules such as proteins but also for peptides, as it is more resistant to contaminants such as detergents and salts. Conversely, CHCA is more frequently utilised for the analysis of smaller analytes such as tryptic peptides and small organic molecules, as it provides higher resolution.

The standard use of ESI-MS is coupled to liquid chromatography (LC) systems; however, ESI MS analysis has been demonstrated in workflows coupled with PCR assays for the identification of infectious diseases. An initial PCR is performed, typically using a multiplex panel of primers to maximise the identification potential of the microorganisms, using conserved regions of DNA. Following amplification, the PCR products undergo desalting, followed by injection to the mass spectrometer. Negative ion mode is used for analysis of oligonucleotide ions, formed by the amplified DNA sequences, which are matched to amplicon reference databases.(28) The development of a clinically approved PCR/ESI-MS was abandoned in 2017, with high reagent costs of $200-300 USD per test, low sample throughput, ultimately being surpassed by the success of MALDI biotyping.(29)

1.3 Tandem Mass Spectrometry for Protein Identification

Tandem MS (MS/MS) utilises two or more mass analysers, which allows fragmentation of analytes and the detection/identification of these fragment ions, assisting in structural elucidation. Fragmentation of peptides/proteins follows basic principles which are dependent on the form of fragmentation used, leading to breakage at specific regions of the peptide/protein chain. Different types of fragment ions are formed from cleavage of the peptide backbone; a, b and c ions are formed when the charge(s) is(are) retained at the N-terminus, and x, y and z ions result from cleavage when the charge(s) is(are) retained at the C-terminus of the peptide/protein chain. The use of collision induced dissociation (CID) forms mostly b and y ions, whereas electron transport dissociation/electron capture

dissociation (ETD/ECD) mostly produces c and z type ions (Figure 1).

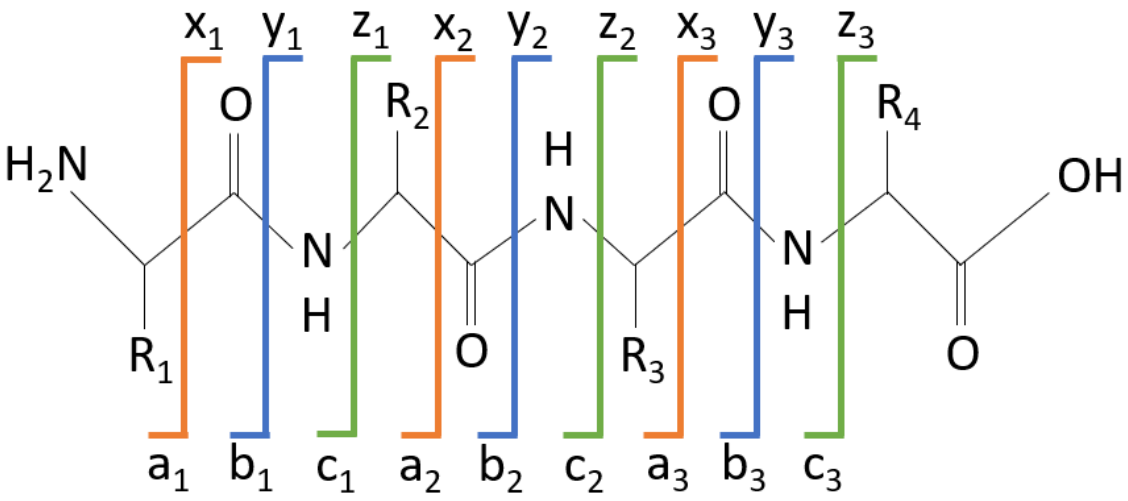

Figure 1 - Fragmentation patterns of proteins and the fragment ions formed following MS/MS.

For the purposes of this thesis, where MS/MS is discussed, it will be focused on the use of CID unless otherwise stated. With CID, analytes collide with a neutral collision gas, such as helium or argon, which dissociates the peptide backbone to give rise to specific fragment ions. Depending on the quality of the spectra and the type of analyte, the mass spectrum of fragment ions can be interpreted manually via mass differences corresponding to amino acids, or automatic searches can be performed. These searches can be performed against widely populated databases, such as Uniprot or Swissprot, or against user-made libraries. Protein fragmentation can be performed 'bottom-up', whereby the protein is digested into smaller peptides, or 'top-down', where the intact protein is fragmented. (Figure 2) Top-down proteomics can allow for identification of post-translational modifications, as well as a reduction in sample preparation time due to the lack of enzymatic digestion.(30) The use of MS/MS is arguably more accurate for protein identification than simply 'mass matching' which occurs in traditional MALDI. MS/MS can be performed with either ESI or MALDI, however ESI is typically preferred. This is due to several reasons, including ion signal

stability, which is often lacking in MALDI, as well as the acquisition of multiply charged ions, which enhances fragmentation spectra and allows the use of a wider range of high-performing mass analysers.(31)

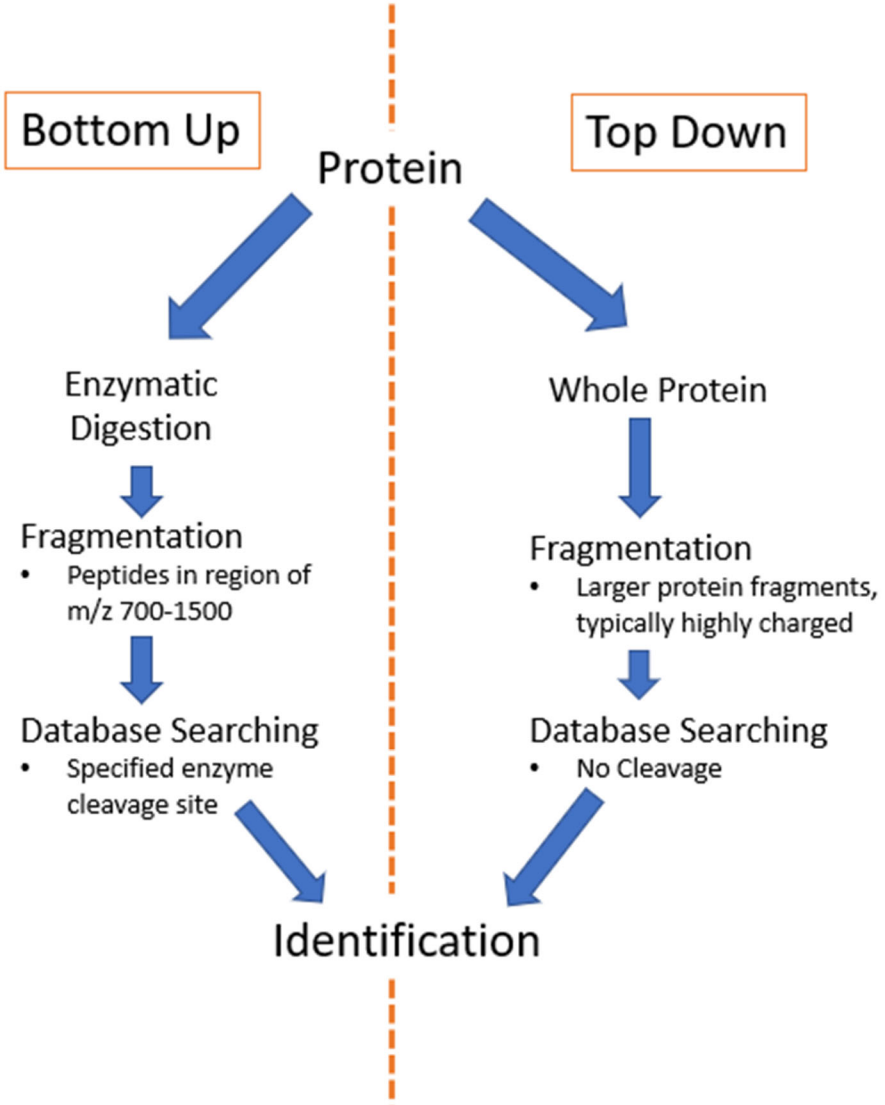

Figure 2 – Typical workflow for the analysis of proteins using MS via a 'bottom-up' or 'top-down' approach.

1.4 Emerging Mass Spectrometry Techniques for Microbial Analysis

ESI MS was first used in the analysis of bacterial lipids in 1995, in particular phospholipids.(32) The analysis of lipids forms the basis of many ambient ionisation techniques that have emerged in the last two decades.(33, 34, 35) With ambient ionisation, ions are formed outside the MS, without additional, or minimal sample preparation

required. Rapid evaporative ionisation mass spectrometry (REIMS) was first developed for tissue analysis during surgery, also known as the "iKnife", and has been widely successful in the discrimination between cancerous and healthy tissue samples.(36) Since its inception in 2009, it has also been used in food research to investigate food safety(37) and fraud.(38) A high-throughput REIMS platform has also been optimised for microbial identification. Initial data for microbial identification allowed species differentiation based upon unique lipidomic MS profiles in negative ion mode, relying upon supervised multivariate statistical analysis for identification.(39) The automated, high-throughput system now allows detection of microorganisms in both positive and negative ion mode, with identification accuracies of 99% and 98.5% respectively.(40) Again, little sample preparation is required, with the harvesting of biological material performed via a handheld probe, or with the HT system, a robotic arm. The HT automated system is said to be capable of analysing 3,000-4,000 colonies over 24 h. (41)

1.5 Veterinary Diagnostics

The use of mass spectrometry, as well as molecular methods,(42) has been extended to veterinary diagnostics with some success. MALDI-TOF MS biotyping for veterinary diagnostics follows the same workflow as in a clinical laboratory for human pathogens, via isolating the microorganism of interest following culture, and subsequent proteomic profiling. However, the breadth of veterinary pathogens in protein databases is limited, in turn limiting the identifying power of protein-based MS analyses in veterinary laboratories. Additional pressure for veterinary diagnostics is sample collection; it is arguably more important than for humans to have non-invasive sample collection techniques, due to animals being in the field rather than within the clinic and potentially less cooperative. Bovine milk has been widely investigated for identification of disease, in particular bovine

mastitis. LAP-MALDI MS has been successfully applied for the detection of sub-clinical mastitis up to two days before clinical presentation, using a non-invasive biofluid and a simple one pot extraction method for biomarker detection.(43) Bovine mastitis is commonly caused by *Staphylococcus aureus* and *Escherichia coli* infection, whereby proteins from these bacteria can be observed in the mass spectrum following protein extraction.(44) Bovine tuberculosis, another example, is a microbial infectious disease caused by *Mycobacterium bovis*. In contrast to mastitis, which quickly presents clinical symptoms of infection, bTB is a latent disease, and *M. bovis* can remain dormant in the body and remain asymptomatic within the animal. In the UK, any detection of *M. bovis* within cattle results in culling to minimise transmission between animals, so effective diagnosis is essential. The use of mass spectrometry and PCR-based testing has been used to identify *M. bovis* infection, however only following microbiological culture, which takes 90 days.(45)

1.6 Antimicrobial Resistance (AMR)

The use of antibiotics over the last 8 decades has put selective pressure on microorganisms, resulting in the emergence of antimicrobial-resistant pathogens. In 2019, The Lancet reported that the leading cause of human death globally was infection with antibiotic-resistant pathogens.(46) Some pathogens are of higher clinical importance than others, in particular the group of ESKAPE pathogens, which are deemed to pose a global threat to human health.(47) Standardised methods of AMR detection include phenotypic methods, such as disc diffusion assays and broth dilution methods.(48) These both measure the level of growth of pathogens in the presence of antibiotics, which can then be compared to official breakpoints to determine levels of susceptibility/resistance to antibiotics. Automated antimicrobial susceptibility testing (AST) systems have been developed, utilising these phenotypic methods, whilst reducing the need for hands-on sample preparation and

increasing throughput.(49) The VITEK systems are based upon growth assays in the presence of antibiotics, via analysis of the turbidity of cultures.(50) RT-PCR can also be used for AMR detection, detecting specific genes responsible for resistance, such as the *mecA* gene responsible for resistance in many MRSAs.(51) PCR-based assays often do not need additional culture or purification, and produce results within as little as 5 h.(52) However, PCR assays incur high reagent costs, and can only detect known genetic sequences. This is an inherent issue, which is also applicable to microbial identification, therefore requiring a prior presumptive identification of the microorganism or resistance mechanism.

Commercially available methods using MS have been developed, based upon hydrolysis of the antibiotic. The MBT-STAR-BL assay detects the presence of a hydrolysed antibiotic following incubation of a β-lactam antibiotic, using the MALDI Biotyper system.(53) Specific non-antibiotic biomarkers can also be detected that are indicative of resistance.(54) So far, there is no single assay using MS that is leading in the clinical laboratory, and therefore there is much room for advancement with novel methods.

1.7 Liquid Atmospheric Pressure (LAP)-MALDI MS

LAP-MALDI is a novel ionisation technique that has been demonstrated for the analysis of biological material and offers many benefits from both ESI and MALDI.(55) As initially reported, a purpose-built atmospheric pressure MALDI source includes the use of a heated inlet capillary directed at a vertically mounted target plate holder, with the laser directed at the sample spot. Counter-flow gas can be applied to assist in desolvation of ions (Figure 3).

Further details of the instrumental set-up can be found elsewhere.(56)

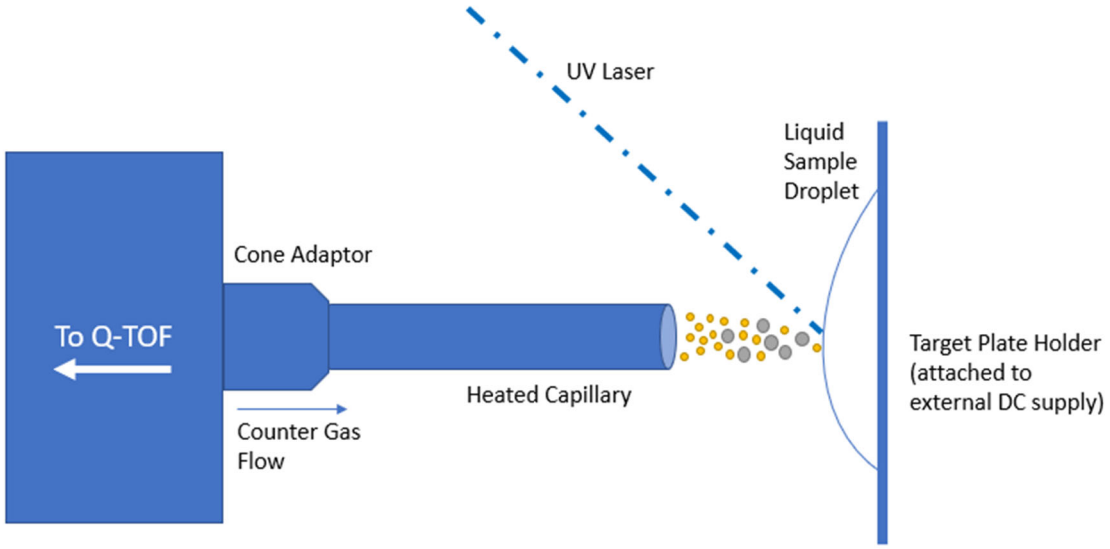

Figure 3 – Schematic of the AP-MALDI source, which is coupled to a Synapt G2-Si and target plate containing a liquid sample droplet.

The use of an atmospheric pressure ion source allows the use of liquid samples, which typically are not used in vacuum conditions due stability of the sample droplet. A liquid matrix is used in LAP-MALDI MS, which is formed of a conventional MALDI matrix chromophore, such as CHCA or DHB, in an appropriate solvent system to allow full dissolution of the matrix compound, as well as a support liquid such as glycerol or ethylene glycol. These are termed liquid support matrices and should not be confused with ionic liquid matrices. In comparison to LSMs as described above, ionic liquid matrices are mixtures of a MALDI matrix compound and an organic base.(57) Ablation of minute volumes of a liquid droplet in LAP-MALDI allows the detection of multiply charged ions for analytes such as peptides and proteins, as observed with ESI. Compared to solid MALDI, the liquid droplet contains a more homogenous distribution of matrix and analyte and a more defined

and consistent sample volume ablation, and therefore produces a more stable ion signal, avoiding the need to find the 'sweet-spot' as required in traditional solid MALDI.

LAP-MALDI MS has been successfully applied in the analysis of biological samples, most notably the analysis of cattle milk in the diagnosis of pre-clinical mastitis.(43) A simple two-step one-pot extraction can be performed to produce a sample for obtaining a LAP-MALDI MS profile containing lipids, peptides, and proteins. The acquisition of a profile spectrum possessing both lipids and proteins is not often performed, due to the mass ranges analysed in both ESI and MALDI. In conventional MALDI MS biotyping, the m/z range below 2,000 is typically excluded due to the interference of matrix cluster ions, particularly with CHCA.(58) It is in this m/z range that lipids are mostly detected. The combination of lipids and peptide/proteins in the same spectrum fundamentally provides higher identification power to LAP-MALDI MS compared to conventional axial-TOF MALDI MS biotyping.

It is the success from the analysis of biological fluids that has prompted the investigation of LAP-MALDI MS to the application of microbial analysis. The use of LAP-MALDI MS could provide additional benefits further to those offered by MALDI biotyping instruments that are currently approved for use. Not only the advantages of LAP-MALDI as described above, but also the potential for superior MS/MS sequencing of proteins. All data acquired in this thesis has been via the use of a LAP-MALDI source coupled to a Synapt G2-Si mass spectrometer, which not only has quadrupole selectivity, but also ion mobility, providing further separation potential. A further continuation into veterinary diagnostics is also explored in this thesis, focusing on bovine tuberculosis in collaboration with the Department for Environment, Food and Rural Affairs (DEFRA).

1.8 Aims of the Research

The aims of this thesis are detailed as follows:

- The **profiling and biotyping of bacteria**, to rival what can be achieved by MALDI-TOF MS in clinical laboratories, following on from the successes of LAP-MALDI MS for the detection of biomolecules, such as lipids and proteins in the same spectrum.
- **Exploration of the MS/MS capabilities** of the setup, providing further definitive analysis of proteins, and therefore bacterial species. The coupling of the LAP-MALDI source to a high performing mass spectrometer like the Synapt G2-Si, also means further tools can be exploited, including ion mobility separation. The use of ion mobility filtering can aid in reducing signal to noise ratio, by selecting for only ions of interest according to their drift time.
- The **detection of multi-drug resistance** in pure bacterial cultures was investigated for further developments in the use of lipid profiles for bacterial identification, alongside an antibiotic hydrolysis assay to measure antimicrobial resistance/susceptibility.
- Further **investigation of LAP-MALDI MS for clinical samples**. Biological samples were also obtained from cattle in order to investigate the detection of bovine tuberculosis caused by a specific bacterium, *Mycobacterium bovis*. This aspect of the project exploited all techniques that were initially optimised using bacterial cultures. Due to the nature of *M. bovis,* it was the response to infection rather than direct detection of the microorganism which provided diagnostic power to the method.

Chapter 2 - General Methodologies

The samples and materials used in this thesis are unique to each application. Specific details of thesis can be found in the relevant chapters.

2. 1 Instrumental Setup

All mass spectrometry analysis was performed based on a LAP-MALDI MS instrumental set up as published previously.(56, 59) In brief, the in-built AP-MALDI source was modified from an electrospray source coupled to a Synapt G2-Si (Waters Corporation, Wilmslow, UK) mass spectrometer. For all MS analyses, the source was set at 3kV with 180L/h N_2 counter-flow gas, with a heated capillary inlet tube to promote desolvation, attached a custom-made cone adaptor. The target plate was vertically mounted to an XYZ stage, and a UV laser directed at the sample droplet. Specific laser wavelengths are specified in later chapters. All samples were analysed with a liquid support matrix (LSM) using CHCA as the chromophore, with ethylene glycol (EG) or propylene glycol (PG) as the support liquid. A solvent system of acetonitrile and H_2O (70:30) was used for dissolution of CHCA. Calibration was performed using sodium iodide (2 µg/µL in H_2O:IPA, 1:1), which was spotted 1:1 with a liquid support solution with the absence of chromophore (ACN:H_2O:EG/PG, 7:3:7). The range for calibration was m/z 100-2,000. The laser was directed to the edge of the droplet to obtain optimal ion signal. Acceptance criteria for the calibration was below 5 ppm.

For MS/MS analysis, CID fragmentation was used, with varied trap and transfer collision voltages depending on the application. Low mass (LM) and high mass (HM) resolutions were adjusted appropriately for isolation of precursor ions. MS and MS/MS analysis was acquired in mobility TOF mode.

For data acquisition, MassLynx (versions 4.1 and 4.2) was used. Spectra from all scans were summed for all MS data. For MS/MS data, specific scans were summed to isolate different

fragment ions where different collision voltages have been applied. Where ion mobility filtering was used, this was performed post-acquisition via manipulation of the drift time plots using Driftscope v2.8. Statistical analysis was performed using AMX® Model Builder, a proprietary Waters software. AMX® software allows direct input of MassLynx raw files for data binning and subsequent statistical analysis including principal component analysis (PCA) and linear discriminant analysis (LDA).

Identification of proteins was performed using MASCOT 2.7 software. Due to incompatibility of Waters mobility data files with MASCOT distiller, fragmentation data was deconvoluted using MaxEnt (Waters plug-in) and de-charged peaks were manually selected to curate peak lists for protein identification. The peak lists were searched using MASCOT against the NCBIprot database.

2.2 Health and Safety

Local health and safety rules were followed in all instances. For handling of biological material, risk assessments were written for the handling of bacteria and for the handling of cattle swabs. Conditions of both risk assessments required inactivation of biological material before removal from the biosafety level 2 laboratory prior to LAP-MALDI MS analysis. In all cases ethanol was used as an inactivating agent.

Chapter 3 - Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry

Research Article

Lellman SE, Cramer R. Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Clin Chem Lab Med. 2020;58(6):930-8.

Author Contributions

Lellman SE – Design of experiment, undertaking investigation and analysis, drafting, and editing the manuscript

Cramer R – Conceptualisation, writing, reviewing, and editing the manuscript, Supervision

Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry

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Keywords – Biotyping, lipids, bacteria, speciation, profiling, matrix-assisted laser desorption/ionization (MALDI), mass spectrometry

Abstract

Background – In recent years, mass spectrometry (MS) has been applied to clinical microbial biotyping, exploiting the speed of matrix-assisted laser desorption/ionization (MALDI) in recording microbe-specific MS profiles. More recently, liquid atmospheric pressure (AP) MALDI has been shown to produce extremely stable ion flux from homogenous samples and 'ESI-like' multiply charged ions for larger biomolecules, whilst maintaining the benefits of traditional MALDI including high tolerance to contaminants, low analyte consumption and rapid analysis. These and other advantages of liquid AP-MALDI MS have been explored in this study to investigate its potential in microbial biotyping.

Methods – Genetically diverse bacterial strains were analysed using liquid AP-MALDI MS, including clinically relevant species such as *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Bacterial cultures were subjected to a simple and fast extraction protocol using ethanol and formic acid. Extracts were spotted with a liquid support matrix (LSM) and analysed using a Synapt G2-Si mass spectrometer with an in-house built AP-MALDI source.

Results – Each species produces a unique lipid profile in the m/z range of 400-1100, allowing species discrimination. Traditional (solid) MALDI MS produced spectra containing a high abundance of matrix-related clusters and an absence of lipid peaks. The MS profiles of the bacterial species tested form distinct clusters using principle component analysis (PCA) with a classification accuracy of 98.63% using a PCA-based prediction model.

Conclusions – Liquid AP-MALDI MS profiles can be sufficient to distinguish clinically relevant bacterial pathogens and other bacteria, based on their unique lipid profiles. The analysis of

the lipid MS profiles is typically excluded from commercial instruments approved for clinical diagnostics.

Introduction

Biotyping using mass spectrometry (MS) has proved highly beneficial in many sectors, including the food industry and clinical laboratories, overtaking traditional microbiological methods such as analysis by microscopy and biochemical assays. The crucial limiting factor of these classical methods is time, requiring after initial bacterial culture at least an additional 18 h for a complete identification(1).

The use of MS for bacterial identification is now a vital tool in the clinical laboratory, leading to a vast reduction in identification turnaround time, from 24-48 h to approximately one hour(2) following the growth period. Thus, the length of stay of patients in hospitals, as well as patient mortality, can be reduced, which in turn significantly reduces hospital costs per infection(3, 4) in comparison to traditional methods. Commercial MS instruments approved for clinical use utilize a (MALDI) source coupled to an axial time-of-flight (TOF) mass spectrometer. These instruments analyse the unique peptide/protein microbial fingerprint in the m/z range of 2,000-12,000, which can provide species-level identification (5, 6). The range below m/z 2,000 is typically excluded from these scans due to the interference of matrix related ions, however this also excludes the detection of low molecular weight metabolites and lipids.

Lipids are essential macromolecules within the bacterial cell, being a major component of the phospholipid bilayer of the cell membrane, as well as having roles in storage and signalling pathways. The term ‘lipidomics’ was first coined by Han and Gross in 2003(7), encompassing the study of the cellular lipidome of biological samples, including bacterial cells. Most commonly, the analytical study of bacterial lipids is performed using pyrolysis MS (8-10), and more recently electrospray ionization (ESI) MS (11, 12). However there has also

been research performed using MALDI-TOF MS (13). These and other MS techniques have demonstrated that certain strains can be distinguished based on their lipid profiles, lending the field of lipidomics to bacterial classification and identification (14, 15). However, only a few studies have explored the use of bacterial lipid profiles for clinical biotyping, and even less by using MALDI MS (16, 17).

Recent developments in liquid AP-MALDI have shown this technique to provide benefits beyond those of traditional MALDI, which is performed with solid samples under vacuum conditions. Liquid MALDI samples are typically comprised of a liquid support matrix (LSM), formed of matrix chromophore molecules and the addition of a viscous support liquid such as glycerol. Liquid MALDI samples have self-healing properties, which allow for a relatively stable ion flux with relatively low sample ablation (18). Liquid MALDI samples also have a greater homogenous distribution of matrix and analyte molecules than solid MALDI samples (19), enabling prolonged analyte ion detection and removing the need for the user to find a 'sweet-spot' on the sample for sufficient analyte ionization which is often needed with solid MALDI samples.

Another major advantage of the use of liquid AP-MALDI is the production of multiply charged ions, which is usually only obtained with an ESI source. This offers greater choice for MS/MS analysis, including ETD/ECD, and provides low m/z values for high-molecular weight biomolecules, thus facilitating the use of conventional, high-performing, hybrid ESI MS instrumentation. The mechanism behind the formation of multiply charged ions has been discussed elsewhere (20-22). However, despite having shown that with the addition of divalent metal cations liquid AP-MALDI can produce doubly charged lipids, diagnostically informative lipid profiles are easier obtained with singly charged lipids as shown in the

analysis of biological samples such as milk extracts (23). The current main limitation of liquid AP-MALDI MS lies in the routine analysis of larger peptides and proteins above 30kDa from complex biological samples (24, 25).

This study demonstrates the novel application of liquid AP-MALDI for the profiling of bacterial lipids to provide species-level identification. We demonstrate that bacteria can be identified using their unique lipid mass fingerprint, providing a rapid analytical alternative for bacterial identification, which in clinical analysis is mostly performed using a peptidomic/proteomic mass fingerprint. The benefits of using liquid MALDI samples over solid MALDI samples to analyse bacterial extracts are demonstrated. Bacterial extracts were prepared using a simple ethanol/formic acid extraction protocol similar to clinical biotyping protocols for solid MALDI MS (26). Secondly, several bacterial species were analysed to obtain a unique lipid profile for each bacterium to allow for species differentiation. These data were used to perform principle component analysis (PCA) to determine the differences between the MS profiles obtained for each bacterial species.

Materials and Methods

2.1 Materials

All MALDI matrix components and protein standards were purchased from Sigma-Aldrich (Gillingham, UK), besides porcine insulin which was purchased from VWR (Leighton Buzzard, UK). The peptide standard mixture was purchased from Bruker UK Ltd. (Coventry, UK). Water, formic acid and acetonitrile (all HPLC grade), as well as ethanol (reagent grade) were purchased from Fisher Scientific (Loughborough, UK).

2.2 Sample preparation

First-generation bacterial strains (Pro-Lab Diagnostics, Merseyside, UK) were received in freeze-dried discs and revived according to recommended growth conditions from Public Health England which were obtained by searching for strain number (see Supplemental Material, Table S1) in the National Collection of Type Cultures (27). All culture media were obtained from Oxoid/ThermoFisher (Basingstoke, UK) and prepared according to manufacturer instructions, including autoclaving at 121˚C for 15 min to ensure sterility.

Following incubation at 37°C for 24 h (or 72 h in the case of *L. brevis*), bacterial growth was scraped off the surface of the media and resuspended in 300µL of HPLC grade water. A volume of 900µL of ethanol was added and mixed by pipetting. The suspension was centrifuged for 2 min at 13,000 rpm, and the supernatant decanted and discarded. Further centrifugation for 2 min at 13,000 rpm was performed, and the supernatant removed by pipetting. The resultant pellet was resuspended in 30µL of 70% formic acid, followed by an equal volume of acetonitrile. The suspension was mixed by pipetting, and then centrifuged for 2 min at 13,000 rpm. Finally, the supernatant was collected and used for AP-MALDI analysis.

An in-house made protein standard mixture consisting of equal concentrations (250pmol/µL) of bovine cytochrome C, bovine ubiquitin, equine myoglobin and porcine insulin was run by liquid MALDI MS prior to each experiment to ensure the instrument was satisfactorily working. The peptide standard mixture was prepared as per manufacturer's guidelines and used as a standard for both solid and liquid MALDI MS analysis.

An ethylene glycol-based LSM was used for all liquid MALDI samples. This was prepared using a 25mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB) solution in acetonitrile and water (70:30%, v/v), using vortexing and sonication to

dissolve the matrix chromophore crystals, followed by the addition of 70% ethylene glycol and vortexing. Liquid MALDI samples were spotted onto a stainless-steel target plate, starting with 0.5µL of LSM solution, then adding 0.5µL of the analyte solution (bacterial extract, protein standard or peptide standard).

For solid MALDI, a CHCA matrix solution was prepared at a concentration of 15mg/mL using acetonitrile and water (70:30%, v/v) with 0.1% trifluoroacetic acid as solvent. Similarly, a DHB matrix solution was prepared at a concentration of 25mg/mL using the same solvent. Solid MALDI samples were spotted onto the stainless-steel target plate, starting with 1µL of analyte solution followed by 1µL of matrix solution. The solid MALDI sample was left to dry at room temperature prior to analysis.

For calibrating the time-of-flight (TOF) mass analyser, a liquid sample droplet containing 0.5µL sodium iodide solution (2µg/mL in 1:1 isopropanol:H_2O, v:v) and 0.5µL of a solution of water, acetonitrile and ethylene glycol (3:7:6, v:v:v) was prepared. Calibration was performed using sodium iodide over the m/z range of 100-2000.

2.3 Liquid AP-MALDI MS Analysis

All MS data were acquired on a Synapt G2-Si (Waters, Wilmslow, UK) in positive TOF mode coupled to an in-house built MALDI source. Details of the instrumental setup have been described in a previous report (20). A potential of +3kV was applied between the target plate and heated transfer tube. The ion source was operated at 3.5kV, with 180L/h N_2 counter-gas flow. A pulsed nitrogen laser (337nm wavelength, 3ns pulse duration) was used at a laser pulse repetition rate of 10Hz at 18µJ/pulse.

Collision-induced dissociation (CID) was used for MS/MS analysis with argon as the collision gas. MS/MS data was acquired using a trap collision energy of 30 V (28).

2.4 MS Data Analysis

All data were processed using MassLynx 4.1® (Waters) software. AMX Model Builder® (Waters) was used to perform PCA on the data. Deconvolution of mass spectral data was performed by UniDec software (29).

2.5 Ethical Approval

Ethical approval was not required for this study as the research undertaken did not involve human or animal material.

3. Results

3.1 Comparison of Liquid and Solid MALDI MS Analysis of Bacterial Extracts

A single bacterial extract was selected for MS analysis with both a solid MALDI sample and a liquid MALDI sample. Figure 1A shows the spectrum obtained for *Escherichia coli* following MS analysis from a solid MALDI sample. The spectrum contains an abundance of high-intensity MALDI matrix-related ions, found in clusters spaced 211 Da apart, decreasing in intensity as the m/z value increases. The mass difference of 211 Da can be attributed to the addition of a sodiated deprotonated CHCA molecule. In general, cluster matrix ion signals for CHCA are typically formed as a result of sodium and potassium ions in varying compositions as previously noted by Smirnov *et al.* (30).

MALDI MS analysis of lipids typically employs matrix chromophore compounds such as DHB (31, 32) or 9-aminoacridine (33-35) rather than CHCA. Thus, the bacterial extracts were also analysed by AP-MALDI MS using DHB (Supplemental Material, Figure S2). However, no improvement was obtained in solid AP-MALDI MS, with the DHB cluster ions being the dominant ion signals, while the mass spectra obtained from DHB-based liquid MALDI samples revealed lower abundance and less variety for lipid ion signals and no ion signals for peptides or proteins when compared to the spectra obtained from CHCA-based liquid MALDI samples.

Spectra of the peptide calibration standard were also recorded from solid MALDI samples to demonstrate the efficacy of solid AP-MALDI on the MS instrument used for this study (Supplemental Material, Figure S1). All peptides that were expected to be detected within the given m/z range of up to 2000 were recorded as singly charged ions using solid AP-MALDI. No multiply charged ions of these or higher molecular mass peptides were detected. However, singly charged lipids from the bacterial extracts were not detected by solid AP-MALDI MS.

On the other hand, when *E. coli* is analysed using a liquid MALDI sample, the resultant spectrum contains a unique fingerprint of lipids in the m/z range of 500-850. Above m/z 850, five peaks from multiply charged ions are present in the spectrum. Deconvolution of these peaks indicate these are the $[M+8H]^{8+}$, $[M+7H]^{7+}$, $[M+6H]^{6+}$, $[M+5H]^{5+}$and $[M+4H]^{4+}$ ions from the same protein or protein fragment, with a software-calculated average mass of 7180 Da.

3.2 Liquid AP-MALDI MS Profiling of Multiple Bacterial Strains

A selection of 10 bacterial species were analysed using liquid AP-MALDI, including *Escherichia coli*, *Klebsiella pneumoniae*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Lactobacillus brevis*, *Enterococcus faecalis*, *Enterococcus hirae* and *Pseudomonas aeruginosa*. Typical mass spectra from their analysis are shown in Figure 2. Visual inspection of these spectra already shows that each species possesses a unique lipid profile in the m/z range of 400-1100. However, many peaks observed in this region are common to multiple bacterial species albeit in varying relative abundances. Putative identification of the lipid ion peaks has been performed using the open access LIPID MAPS structure database (Supplemental Material, Table S2) (36).

3.3 MS/MS Analysis of E. coli *Lipid Profile Peaks*

To confirm the suspected identity of putative lipid profile peaks in the selected m/z ranges, MS/MS analysis was performed. Figure 3 shows the MS/MS spectrum of the precursor ion at m/z 726.5, which can be attributed to the sodium adduct of a phosphatidylethanolamine (PE) lipid. Evidence for this identification can be found in the loss of 43 Da (presumably C_2H_5N from the head group), resulting in a peak at m/z 683.5, and the loss of 141 Da (ethanolamine phosphate head group), resulting in a peak at m/z 585.5 - both are characteristic fragments of PEs as reported earlier(37). Other fragmentation detected in the MS/MS spectrum include the putative loss of fatty acid groups C16:0 and C17:1 as seen as a loss of 256 Da and 268 Da, respectively, from the fragment ion at m/z 683.44 (see Figure 3). All of the above peaks have been previously identified by Zhang *et al.* (37).

3.4 Principle Component Analysis

Raw MS profile data files were imported to the AMX Model Builder® software for PCA. For each of the ten species, nine data files acquired from three biological replicates from

separate cultures analysed in triplicates were added to the model, and the m/z range 400-1100 was selected for multivariate statistics. Classification performance of the AMX® model was assessed by the software's built-in 'leave 20% out' cross validation method, reporting a correct classification rate of 97.78%. Figure 4 shows a plot of the PCA data for the first 3 principle components (PC1, PC2 and PC3). Data points for each species form separate clusters, allowing species discrimination. The peaks associated with the highest influence on variation in PC1 and PC2 are tabulated in the Supplemental Material (Table S3).

Discussion

Each bacterial species in this study produced a unique lipid MS profile when analysed using liquid AP-MALDI MS. In clinical MS biotyping instruments, lipid profiles are typically not analysed, as these instruments are optimized for the analysis of the unique peptidomic/proteomic fingerprint of microbial extracts, detecting mainly ribosomal proteins and their fragments in the m/z range of 2,000-12,000. The analytical sensitivity of such instruments is commonly enhanced by using axial TOF mass analyzers in the linear mode with ion deflection devices or lower detector voltages for lowering the detection of the m/z range in which lipids are detected. Thus, lipids are excluded from the analysis in these instruments, arguably due to reasons associated with excessive ion suppression from matrix cluster formation when using solid MALDI and detector saturation in the low m/z range as it is often the case for axial MALDI-TOF instruments operated in the linear mode.

As shown, by using liquid AP-MALDI the lower m/z range is far less populated with high-intensity matrix cluster ions as observed with solid AP-MALDI in this study (see Figure 1) and solid MALDI as reported in the literature (30). *E. coli* extracts analysed by solid AP-MALDI MS only revealed matrix cluster ions, providing no diagnostic information.

The use of liquid AP-MALDI coupled to an orthogonal Q-TOF mass analyzer also overcomes some of the limitation due the potentially excessive desorption of neutral matrix compounds and clusters as well as late matrix ion cluster formation due to sodium- and potassium-mediated cation formation, which can pose far greater detector saturation issues in linear mode axial TOF mass analyzers. Thus, lipids and metabolites occupying the same m/z range as matrix/cation clusters will be easier to detect with orthogonal hybrid instrumentation that can effectively decouple the source from the analyzer for the purpose of limiting the amount of matrix clusters reaching the detector.

Some hybrid MS instrumentation like the one used in this study also allow ion mobility separation, which particularly for lipids can provide another dimension of separation and will be further investigated in future studies. Ion mobility has the capability to separate ions based on their collisional cross section, which can provide the separation of isobaric species (38), as well as a substantial reduction of chemical background noise (39).

As lipids are essential components of the cell membrane, with roles in protein localization which is vital to the bacterial life cycle (40), the detection of their ion signals provides valuable diagnostic information, allowing to distinguish bacteria based on their lipid profile (15, 37).

A diverse set of bacteria were selected for analysis in this study to account for species diversity in demonstrating the use of liquid AP-MALDI MS in bacterial classification for a genetically varied group of organisms. Some of the selected species are of high clinical importance. Amongst these, *S. aureus, K. pneumoniae* and *P. aeruginosa* are part of a group named ESKAPE pathogens, which are organisms that have a high potential to resist actions of antibiotics (41). Closely related species were also included to test how well these can be

distinguished using their lipid profiles. Thus, *S. aureus* and *S. epidermidis*, as well as *E. hirae* and *E. faecalis* were included as these bacteria respectively belong to the same genus and possess relatively similar lipid profiles. Also, a high degree of similarity can be seen between certain species, such as *E. coli* and *K. pneumoniae*, with an almost identical lipid profile. These were distinguishable by the intensities of their lipid ion peaks. As both species are member of the same family, *Enterobacteriaeceae*, the close resemblance of their lipid profiles can be expected. Similar lipid MS profiles have also been observed in other studies for *Enterobacteriaeceae*, with the distribution of PEs significantly varying based on their genus (42). However, here it has been shown that it is possible to achieve species-level discrimination.

A marked difference in the profile pattern can be seen for lipid profiles obtained for gram-positive and gram-negative bacteria. Previous MS studies by Zhang *et al.* have shown that spectra of gram-negative bacteria contain fatty acids, lyso-phospholipids, phosphatidylethanolamines (PE) and phosphatidylglycerols (PG) in abundance, while spectra of gram-positive bacteria contain lipopeptides, and with limited abundance lyso-phospholipids (37). This difference can also be seen in the distribution of the lipid profiles between the gram-positive and gram-negative species used in this study. All gram-positive bacteria investigated (Figure 2E-J) exhibit a lipid profile in the m/z range of 900-1000, whereas gram-negative species show the highest intensity distribution of lipid ion signals in the m/z range of 650-800. This is consistent with MS studies that demonstrate that gram-negative bacteria have a high ion signal abundance of PEs and PGs within this range (43). Gram-positive bacteria exhibit a lipid profile in a higher m/z range, due to the presence of different lipid classes, such as lysophospholipids present in *S. aureus* (44) and cardiolipins found in *Lactobacillus* species (45).

It is not only the lipid profile that can be potentially exploited using liquid AP-MALDI MS. In Figure 1B, the higher m/z range shows that multiply charged ions can be obtained from bacterial samples. The estimated molecular weight of the underlying molecular species is 7180 Da, possibly a ribosomal protein or protein fragment as typically detected in commercial MALDI MS biotyping instruments (46). The detection of proteins using MALDI and axial-TOF instrumentation is the current standard for mass spectrometry in clinical microbiology, and therefore the acquisition of lipid ion signals alongside peptide and protein ion signals contributes valuable diagnostic information.

In this study, species-level discrimination has been achieved for all investigated bacteria using lipid profiles. However, there might be species that cannot be distinguished based on their lipid MS profile alone. In these cases, the ability of liquid AP-MALDI MS to detect lipid profiles in combination with some multiply charged peptide and protein ions can further improve bacterial biotyping, potentially allowing the discrimination between closely related species and strain-level identification, which could allow tracking of strain evolution and acquisition of resistance profiles.

Abundant ion signals for lipids and proteins in hybrid MS instruments also allow superior MS/MS analysis compared to current commercial MS biotyping instrumentation, thus providing structural information for these macromolecules. As clinical biotyping of microorganisms is performed on axial MALDI-TOF mass spectrometers, MS/MS analysis and therefore bacterial identification is principally inferior. The use of the AP-MALDI source and Q-TOF mass analyzer in this study allows the acquisition of high-quality MS/MS data, providing further highly specific sequence information for peptides and proteins from the same analysis without additional sample preparation. Protocols for optimised

lipid/peptide/protein extraction and the combined lipid profile and sequence analysis are currently under development and will be reported elsewhere.

In summary, this study has investigated the application of liquid AP-MALDI MS on a hybrid Q-TOF MS instrument for the analysis of bacterial extracts. In a clinical laboratory, bacterial identification by mass spectrometry is typically performed based on a unique proteomic fingerprint. The data presented here shows that there is the potential to perform species-level identification based on the unique lipid MS profile of the bacteria, which is typically not obtained from commercial MALDI-TOF instruments approved for clinical analysis. It is important to note that environmental factors can influence the lipid composition in bacteria. Thus, further studies with respect to the influence of the growth medium, temperature and incubation time are needed.

The ability of liquid AP-MALDI to generate multiply charged peptide/protein ions through its application on hybrid high-performing MS/MS instrumentation will undoubtedly add analytical power to the detection of lipid MS profiles. Ultimately, this option should provide bacterial identification of higher confidence compared to current instruments approved for clinical use.

Data supporting the results reported in this paper are openly available from the University of Reading Research Data Archive at http://dx.doi.org/10.17864/1947.221.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by EPSRC through grant EP/R513301/1.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization played no role in the study design; in the collection, analysis, and interpretation of data; in writing of the report; or in the decision to submit the report for publication.

Acknowledgements: Access to the AMX Model Builder® software was kindly provided by Waters Corporation.

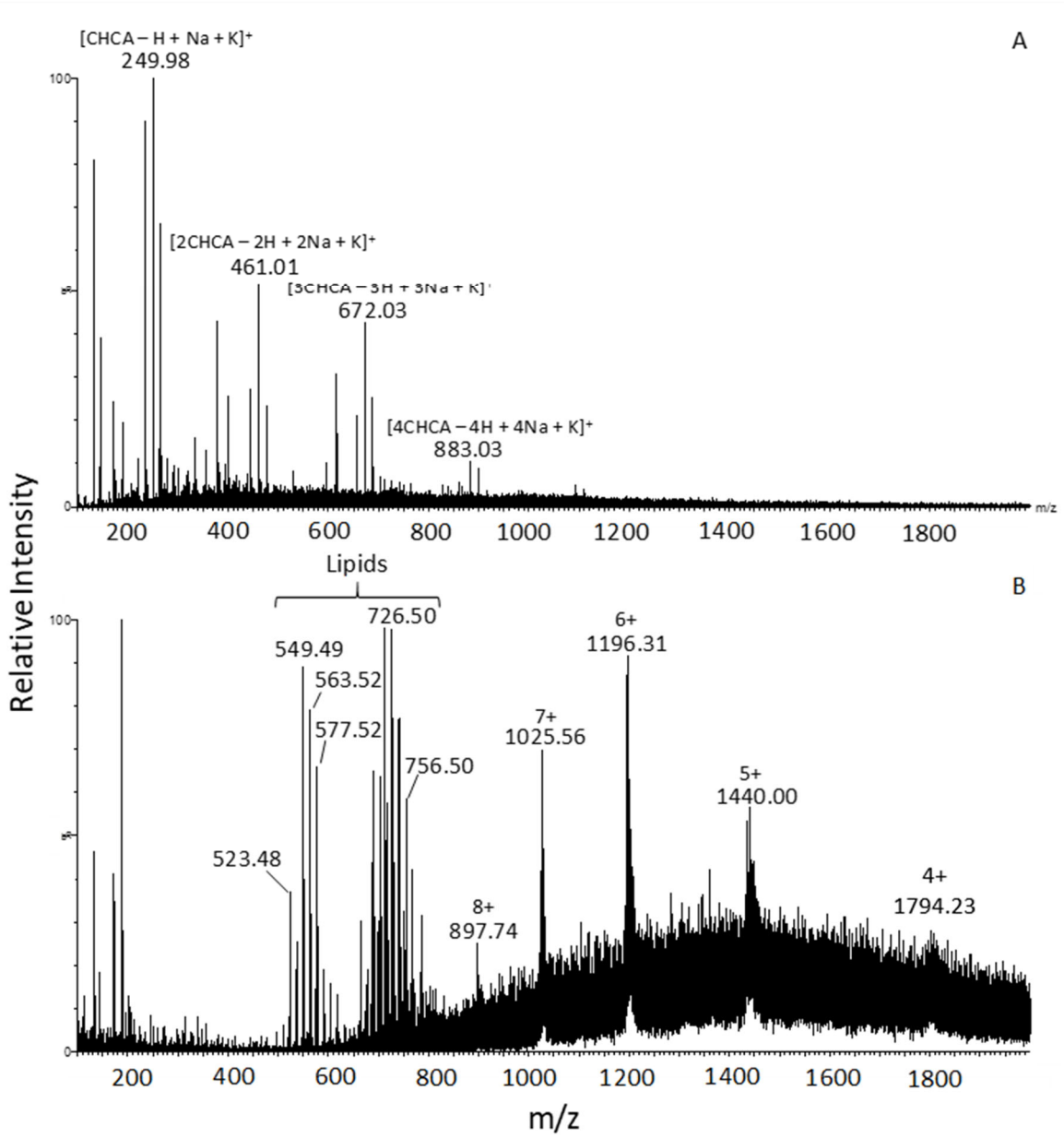

Figure 1 – AP-MALDI mass spectra of E. coli obtained from a solid (A) and liquid (B) MALDI sample, where CHCA represents the matrix chromophore compound. The most abundant isotopologues are labelled.

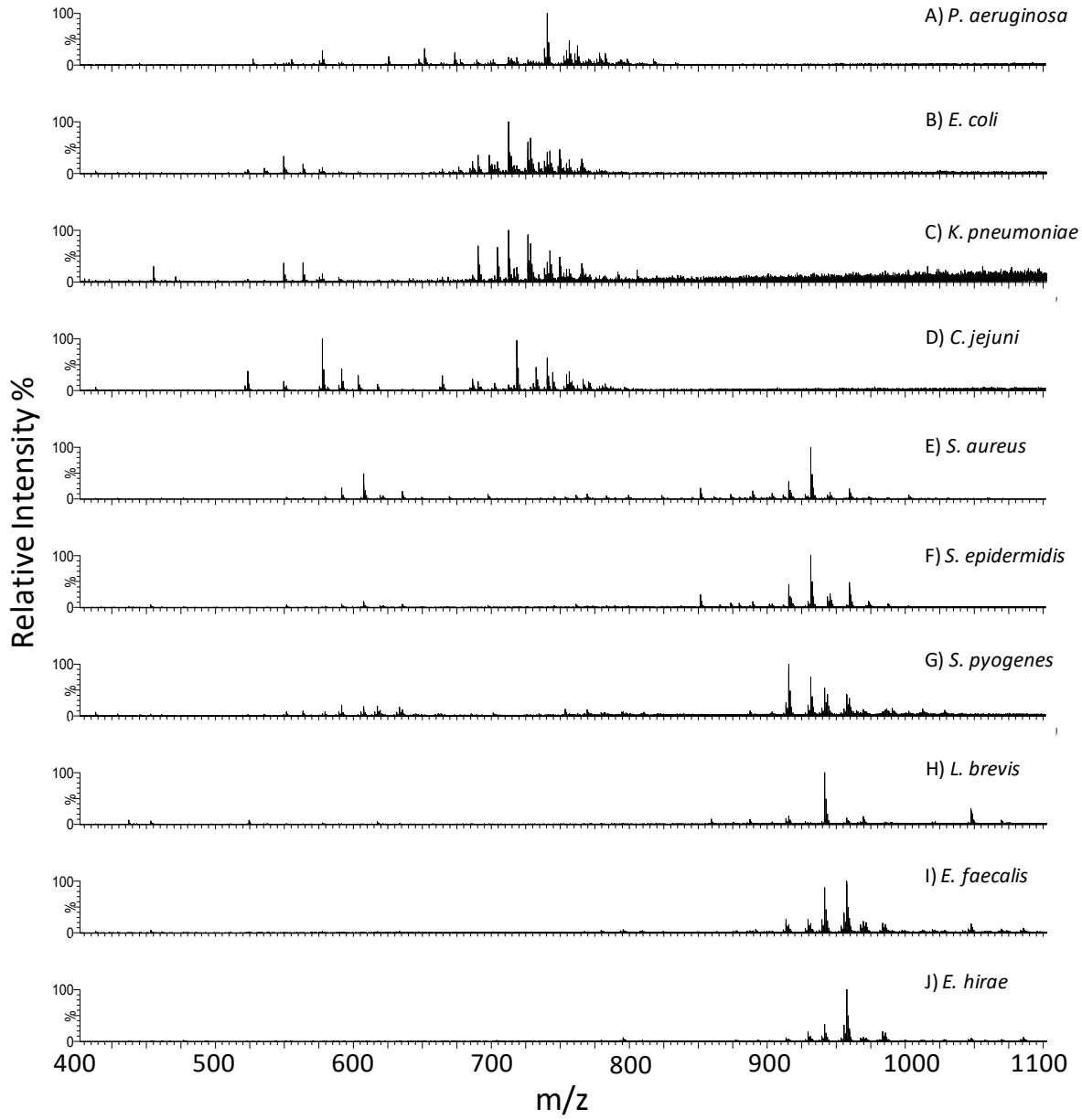

Figure 2 - Liquid AP-MALDI mass spectra of the lipid profiles of several bacterial species in positive ion mode.

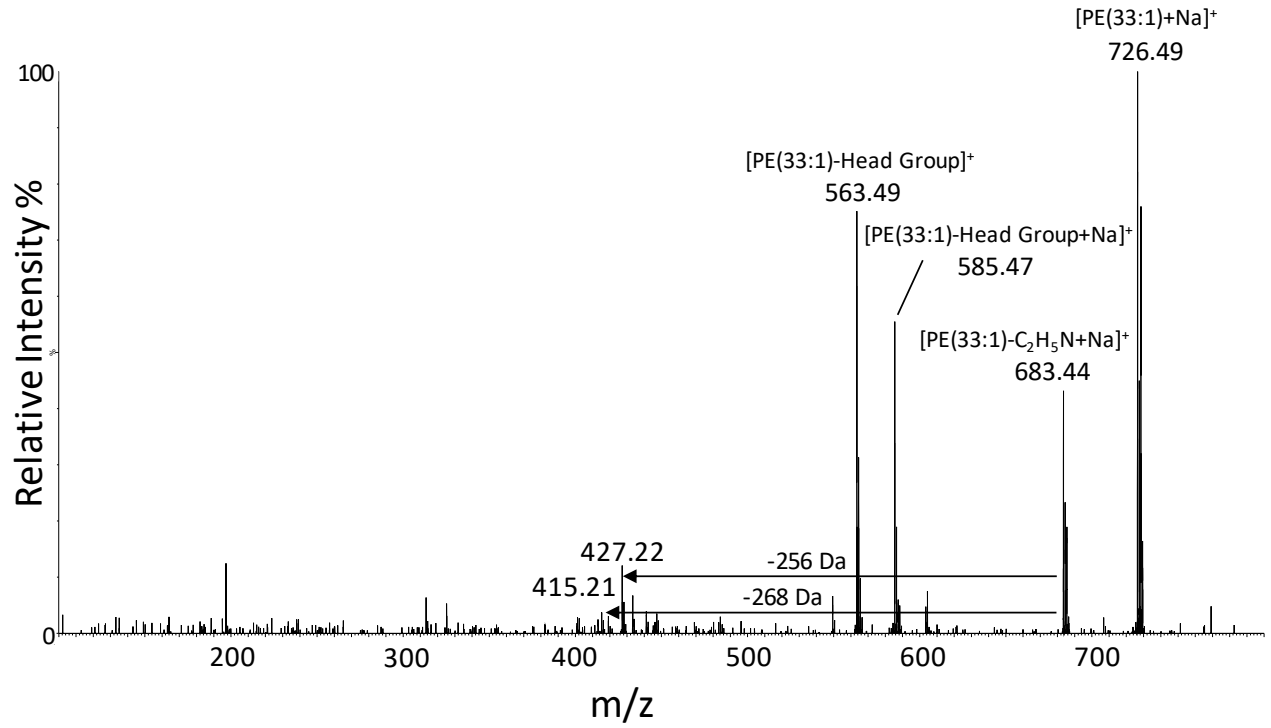

Figure 3 – Liquid AP-MALDI MS/MS spectrum of the precursor ion at m/z 726.5, detected in the lipid MS profile of *E. coli*.

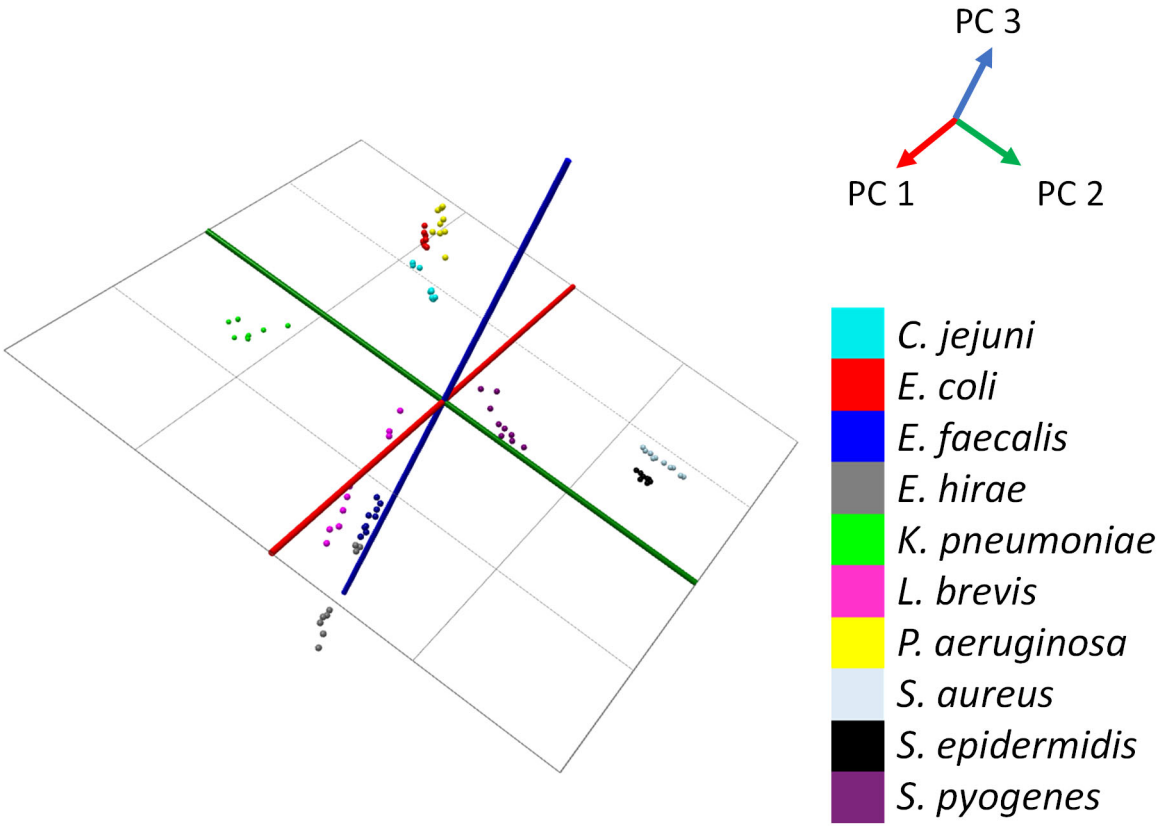

Figure 4 – Plot of the PCA data points for the first three principle components (PC1, PC2 and PC3) using MS profile data from 10 bacterial species.

References

1. Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti J-L, et al. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clinical Biochemistry. 2011;44(1):104-9.
2. Croxatto A, Prod'hom G, Greub G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiology Reviews. 2012;36(2):380-407.
3. Perez KK, Olsen RJ, Musick WL, Cernoch PL, Davis JR, Land GA, et al. Integrating Rapid Pathogen Identification and Antimicrobial Stewardship Significantly Decreases Hospital Costs. Archives of Pathology and Laboratory Medicine. 2013;137(9):1247-54.
4. Patel TS, Kaakeh R, Nagel JL, Newton DW, Stevenson JG. Cost Analysis of Implementing Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Plus Real-Time Antimicrobial Stewardship Intervention for Bloodstream Infections. Journal of Clinical Microbiology. 2017;55(1):60-7.
5. Faron ML, Buchan BW, Hyke J, Madisen N, Lillie JL, Granato PA, et al. Multicenter Evaluation of the Bruker MALDI Biotyper CA System for the Identification of Clinical Aerobic Gram-Negative Bacterial Isolates. PLOS ONE. 2015;10(11):e0141350.
6. Seng P, Drancourt M, Gouriet F, Scola BL, Fournier PE, Rolain JM, et al. Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clinical Infectious Diseases. 2009;49(4):543-51.
7. Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. Journal of Lipid Research. 2003;44(6):1071-9.
8. Dworzanski JP, Berwald L, Meuzelaar HLC. Pyrolytic Methylation-Gas Chromatography of Whole Bacterial Cells for Rapid Profiling of Cellular Fatty Acids. Applied and Environmental Microbiology. 1990;56(6):1717-24.
9. Basile F, Voorhees KJ, Hadfield TL. Microorganism gram-type differentiation based on pyrolysis-mass spectrometry of bacterial Fatty Acid methyl ester extracts. Applied and Environmental Microbiology. 1995;61(4):1534-9.
10. Dworzanski JP, Tripathi A, Snyder AP, Maswdeh WM, Wick CH. Novel biomarkers for Gram-type differentiation of bacteria by pyrolysis–gas chromatography–mass spectrometry. Journal of Analytical and Applied Pyrolysis. 2005;73(1):29-38.
11. Fang J, Barcelona MJ. Structural determination and quantitative analysis of bacterial phospholipids using liquid chromatography/electrospray ionization/mass spectrometry. Journal of Microbiological Methods. 1998;33(1):23-35.
12. Smith PBW. Characterization of Bacterial Phospholipids by Electrospray Ionization Tandem Mass Spectrometry. Analytical Chemistry. 1995;67(11):1824-30.
13. Gidden J, Denson J, Liyanage R, Ivey DM, Lay JO. Lipid Compositions in Escherichia coli and Bacillus subtilis During Growth as Determined by MALDI-TOF and TOF/TOF Mass Spectrometry. International Journal of Mass Spectrometry. 2009;283(1-3):178-84.
14. Hamid AM, Jarmusch AK, Pirro V, Pincus DH, Clay BG, Gervasi G, et al. Rapid Discrimination of Bacteria by Paper Spray Mass Spectrometry. Analytical Chemistry. 2014;86(15):7500-7.
15. Heller DN, Cotter RJ, Fenselau C, Uy OM. Profiling of bacteria by fast atom bombardment mass spectrometry. Analytical Chemistry. 1987;59(23):2806-9.
16. Strittmatter N, Rebec M, Jones EA, Golf O, Abdolrasouli A, Balog J, et al. Characterization and Identification of Clinically Relevant Microorganisms Using Rapid Evaporative Ionization Mass Spectrometry. Analytical Chemistry. 2014;86(13):6555-62.
17. Shu X, Li Y, Liang M, Yang B, Liu C, Wang Y, et al. Rapid lipid profiling of bacteria by online MALDI-TOF mass spectrometry. International Journal of Mass Spectrometry. 2012;321-322:71-6.

18. Cramer R, Pirkl A, Hillenkamp F, Dreisewerd K. Liquid AP-UV-MALDI Enables Stable Ion Yields of Multiply Charged Peptide and Protein Ions for Sensitive Analysis by Mass Spectrometry. Angewandte Chemie International Edition. 2013;52(8):2364-7.
19. Towers MW, Mckendrick JE, Cramer R. Introduction of 4-Chloro-α-cyanocinnamic Acid Liquid Matrices for High Sensitivity UV-MALDI MS. Journal of Proteome Research. 2010;9(4):1931-40.
20. Ryumin P, Brown J, Morris M, Cramer R. Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS. Methods. 2016;104:11-20.
21. Ryumin P, Cramer R. The composition of liquid atmospheric pressure matrix-assisted laser desorption/ionization matrices and its effect on ionization in mass spectrometry. Analytica Chimica Acta. 2018;1013:43-53.
22. Trimpin S, Wang B, Inutan ED, Li J, Lietz CB, Harron A, et al. A Mechanism for Ionization of Nonvolatile Compounds in Mass Spectrometry: Considerations from MALDI and Inlet Ionization. Journal of The American Society for Mass Spectrometry. 2012;23(10):1644-60.
23. Hale OJ, Cramer R. Collision-induced dissociation of doubly-charged barium-cationized lipids generated from liquid samples by atmospheric pressure matrix-assisted laser desorption/ionization provides structurally diagnostic product ions. Analytical and Bioanalytical Chemistry. 2018;410(5):1435-44.
24. Hale OJ, Morris M, Jones B, Reynolds CK, Cramer R. Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Adds Enhanced Functionalities to MALDI MS Profiling for Disease Diagnostics. ACS Omega. 2019;4(7):12759-65.
25. Hale OJ, Ryumin P, Brown JM, Morris M, Cramer R. Production and analysis of multiply charged negative ions by liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Rapid Communications in Mass Spectrometry. 2018;1(8).
26. Bruker Daltonik GmbH. Instructions for Use - MALDI Biotarget 48. Available from: https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/InstructionForUse/IFU_268711_267615_226413_MALDI_Biotarget_48_Rev1.pdf
27. England PH. Culture Collections [National Collection of Type Cultures]. Available from: https://www.phe-culturecollections.org.uk/collections/nctc.aspx.
28. Cramer R, Corless S. The nature of collision-induced dissociation processes of doubly protonated peptides: comparative study for the future use of matrix-assisted laser desorption/ionization on a hybrid quadrupole time-of-flight mass spectrometer in proteomics. Rapid Communications in Mass Spectrometry. 2001;15(22):2058-66.
29. Marty MT, Baldwin AJ, Marklund EG, Hochberg GKA, Benesch JLP, Robinson CV. Bayesian Deconvolution of Mass and Ion Mobility Spectra: From Binary Interactions to Polydisperse Ensembles. Analytical Chemistry. 2015;87(8):4370-6.
30. Smirnov IP, Zhu X, Taylor T, Huang Y, Ross P, Papayanopoulos IA, et al. Suppression of α-Cyano-4-hydroxycinnamic Acid Matrix Clusters and Reduction of Chemical Noise in MALDI-TOF Mass Spectrometry. Analytical Chemistry. 2004;76(10):2958-65.
31. Vieler A, Wilhelm C, Goss R, Süß R, Schiller J. The lipid composition of the unicellular green alga Chlamydomonas reinhardtii and the diatom Cyclotella meneghiniana investigated by MALDI-TOF MS and TLC. Chemistry and Physics of Lipids. 2007;150(2):143-55.
32. Jackson SN, Wang H-YJ, Woods AS. Direct Profiling of Lipid Distribution in Brain Tissue Using MALDI-TOFMS. Analytical Chemistry. 2005;77(14):4523-7.
33. Cerruti CD, Benabdellah F, Laprévote O, Touboul D, Brunelle A. MALDI Imaging and Structural Analysis of Rat Brain Lipid Negative Ions with 9-Aminoacridine Matrix. Analytical Chemistry. 2012;84(5):2164-71.
34. Angelini R, Vitale R, Patil VA, Cocco T, Ludwig B, Greenberg ML, et al. Lipidomics of intact mitochondria by MALDI-TOF/MS. Journal of Lipid Research. 2012;53(7):1417-25.

35. Angelini R, Babudri F, Lobasso S, Corcelli A. MALDI-TOF/MS analysis of archaebacterial lipids in lyophilized membranes dry-mixed with 9-aminoacridine. Journal of Lipid Research. 2010;51(9):2818-25.
36. Structure Database (LMSD) LIPID MAPS (R) Lipidomics Gateway. Available from: https://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=SetupTextOntologySearch.
37. Zhang JI, Talaty N, Costa AB, Xia Y, Tao WA, Bell R, et al. Rapid direct lipid profiling of bacteria using desorption electrospray ionization mass spectrometry. International Journal of Mass Spectrometry. 2011;301(1):37-44.
38. Woods AS, Ugarov M, Egan T, Koomen J, Gillig KJ, Fuhrer K, et al. Lipid/Peptide/Nucleotide Separation with MALDI-Ion Mobility-TOF MS. Analytical Chemistry. 2004;76(8):2187-95.
39. Venne K, Bonneil E, Eng K, Thibault P. Improvement in Peptide Detection for Proteomics Analyses Using NanoLC−MS and High-Field Asymmetry Waveform Ion Mobility Mass Spectrometry. Analytical Chemistry. 2005;77(7):2176-86.
40. Barák I, Muchová K. The role of lipid domains in bacterial cell processes. International Journal Molecular Science. 2013;14(2):4050-65.
41. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. Expert Review of Anti-infective Therapy. 2013;11(3):297-308.
42. Ishida Y, Madonna AJ, Rees JC, Meetani MA, Voorhees KJ. Rapid analysis of intact phospholipids from whole bacterial cells by matrix-assisted laser desorption/ionization mass spectrometry combined with on-probe sample pretreatment. Rapid Communications in Mass Spectrometry. 2002;16(19):1877-82.
43. Ishida Y, Kitagawa K, Nakayama A, Ohtani H. Complementary analysis of lipids in whole bacteria cells by thermally assisted hydrolysis and methylation-GC and MALDI-MS combined with on-probe sample pretreatment. Journal of Analytical and Applied Pyrolysis. 2006;77(2):116-20.
44. Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, et al. Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. Journal of Experimental Medicine. 2001;193(9):1067-76.
45. Calvano CD, Zambonin CG, Palmisano F. Lipid fingerprinting of Gram-positive lactobacilli by intact cells – matrix-assisted laser desorption/ionization mass spectrometry using a proton sponge based matrix. Rapid Communications in Mass Spectrometry. 2011;25(12):1757-64.
46. Freiwald A, Sauer S. Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols. 2009;4(5):732.

Supplemental Material for

"Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry"

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Table S1 – Bacterial species and NCTC strain number used for liquid AP-MALDI MS analysis.

Species	**NCTC Strain Number**
Campylobacter jejuni	11322
Escherichia coli	12241
Enterococcus faecalis	775
Enterococcus hirae	5855
Klebsiella pneumoniae	9633
Lactobacillus brevis	13386
Pseudomonas aeruginosa	12903
Staphylococcus aureus	6571
Staphylococcus epidermidis	13360
Streptococcus pyogenes	12696

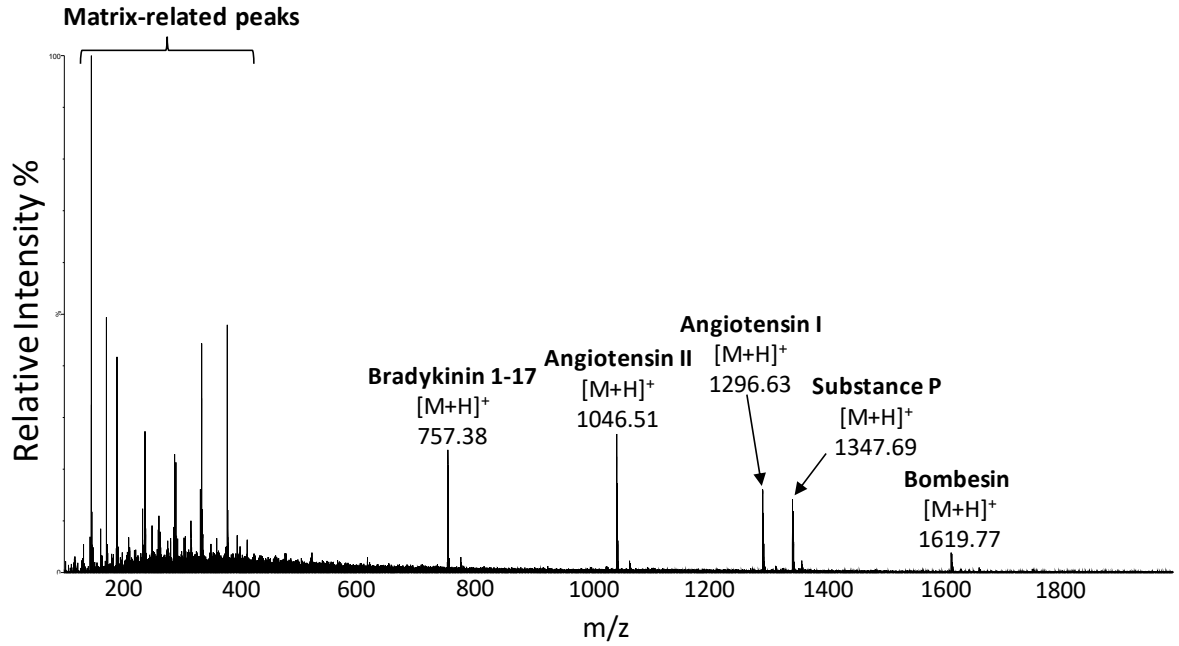

Figure S1 – Solid AP-MALDI mass spectrum of the peptide calibration standard mixture used in this study.

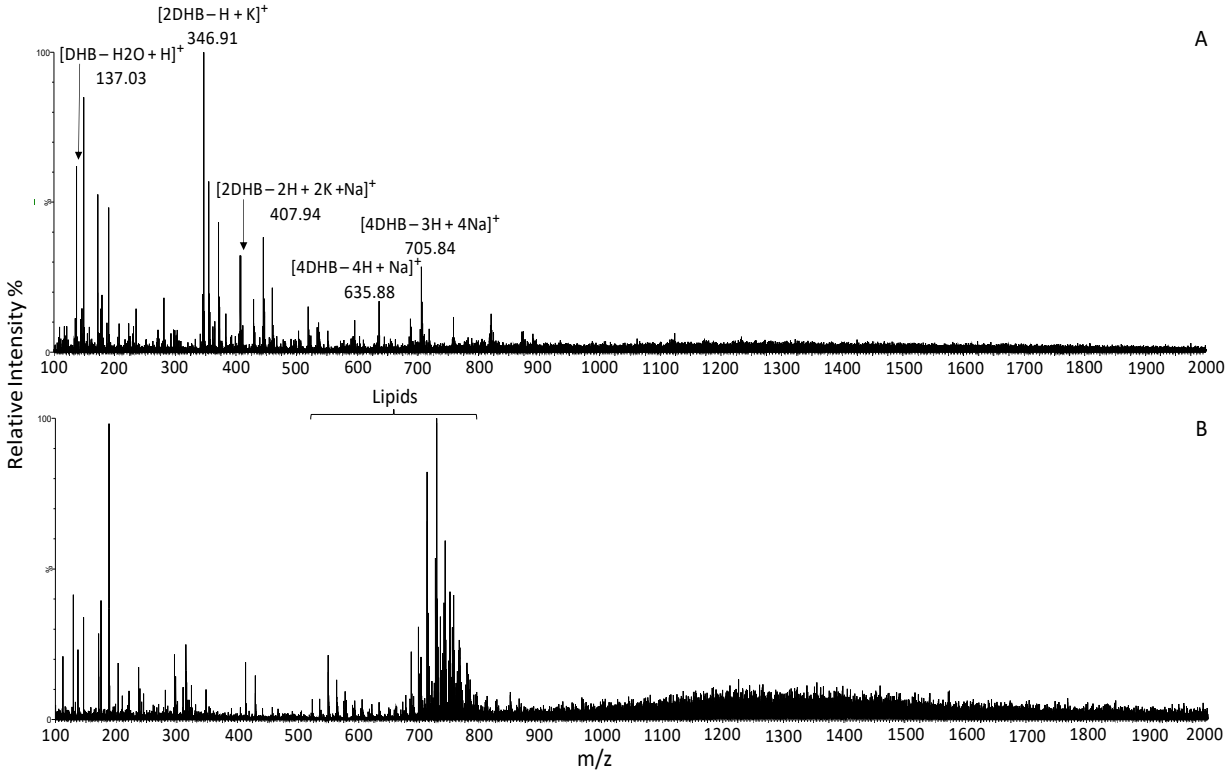

Figure S2 – AP-MALDI mass spectra of *E. coli* obtained from a solid (A) and liquid (B) MALDI sample, using DHB as the matrix chromophore compound.

Table S2 – Putative identification of lipid species observed in liquid AP-MALDI MS profiling of bacterial species (as seen in Figure 2), using accurate mass matching of entries in the LIPID MAPS Structure Database (LMSD; https://www.lipidmaps.org/data/structure/LMSDSearch.php).

m/z*	*C. jejuni*	*E. coli*	*E. faecalis*	*E. hirae*	*K. pneumoniae*	*L. brevis*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*S. pyogenes*	Elemental Composition#	Putative Lipid Assignment$	LIPID MAPS Structure Database Accession Number
523.47	•	•			•						$C_{32}H_{58}O_5$	Diadylglycerol	LMGL02010334
535.47		•									$C_{33}H_{58}O_5$	Diadylglycerol or Isoprenoid	LMGL02010338, LMPR01070021, LMPR01070087, LMPR01070088, LMPR01070164
549.47	•	•			•						$C_{38}H_{60}O_2$	Fatty Ester	LMFA07011032
563.50		•			•		•				$C_{35}H_{62}O_2$	Diadylglycerol	LMGL02010343, LMGL02010386, LMGL02010409, LMGL02010454
577.51	•	•	•	•	•	•	•				$C_{37}H_{68}O_4$	Fatty Alcohol	LMFA05000691
589.45		•	•	•	•						$C_{37}H_{64}O_5$	Diadylglycerol	LMGL02010028, LMGL02010035, LMGL02010351, LMGL02010392, LMGL02010415, LMGL02010475, LMGL02010498
591.49	•				•			•	•	•	$C_{37}H_{66}O_5$	Diadylglycerol	LMGL02010024, LMGL02010031, LMGL02010032, LMGL02010350, LMGL02010391, LMGL02010414, LMGL02010474
603.53	•	•			•						$C_{39}H_{70}O_4$	Diadylglycerol	LMGL02070004, LMGL02070010, LMGL02070011, LMGL02070023, LMGL02070024
607.46								•	•	•	$C_{37}H_{68}O_4$	Glycerophosphate	LMGP10020004, LMGP10020019
651.58							•				$C_{41}H_{78}O_5$	Diadylglycerol or Sterols	LMGL02010081, LMGL02010082, LMGL02010105, LMGL02010106, LMGL02010113, LMGL02070009, LMGL02070018, LMGL02070020, LMGL02070031, LMGL02070032, LMST01020003, LMST01020054, LMST01020056, LMST01020094
664.48	•	•									$C_{35}H_{70}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010700, LMGP01011244, LMGP01011262, LMGP01011317, LMGP01011339, LMGP01011363, LMGP01011409, LMGP01020176, LMGP02010106, LMGP02010297, LMGP02010302, LMGP02010316, LMGP02011207, LMGP02011215, LMGP02011255, LMGP02011261

676.49		•									$C_{36}H_{70}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010392, LMGP01011319, LMGP01011341, LMGP01011364, LMGP01011381, LMGP01011432, LMGP01011473, LMGP02010005, LMGP02010353, LMGP02010355, LMGP02010371, LMGP02010390, LMGP02010410, LMGP02010429, LMGP02010480, LMGP02010507, LMGP02010518, LMGP02010563, LMGP02010636, LMGP02010793, LMGP02011235, LMGP02011267
690.49		•			•						$C_{37}H_{72}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010735, LMGP01011321, LMGP01011343, LMGP01011365, LMGP01011382, LMGP01011411, LMGP01011433, LMGP01011474, LMGP01011521, LMGP02010372, LMGP02010395, LMGP02010432, LMGP02010456, LMGP02010482, LMGP02010520, LMGP02010541, LMGP02010565, LMGP02010622, LMGP02010794, LMGP02010841, LMGP02011199, LMGP02011228, LMGP02011247, LMGP02011268
704.50		•			•						$C_{38}H_{74}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010440, LMGP01010485, LMGP01011344, LMGP01011384, LMGP01011412, LMGP01011435, LMGP01011463, LMGP01011475, LMGP01011522, LMGP01011595, LMGP02010396, LMGP02010414, LMGP02010438, LMGP02010458, LMGP02010485, LMGP02010521, LMGP02010543, LMGP02010567, LMGP02010624, LMGP02010638, LMGP02010769, LMGP02010795, LMGP02010842, LMGP02011226, LMGP02011266
712.48		•					•				$C_{39}H_{70}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01011351, LMGP01011701, LMGP02010380, LMGP02010443, LMGP02010525, LMGP02010526, LMGP02010686, LMGP02010716, LMGP02010744, LMGP02010905, LMGP02010935, LMGP02011102, LMGP02011208, LMGP02011223, LMGP02011242
718.53	•	•	•								$C_{39}H_{76}NO_8P$	Glycerophosphocholine, Glycerophosphoethanolamine or Glycerophosphoserine	LMGP01010002, LMGP01010008, LMGP01010535, LMGP01011328, LMGP01011347, LMGP01011367, LMGP01011386, LMGP01011437, LMGP01011464, LMGP01011477, LMGP01011523, LMGP01011596, LMGP01011756, LMGP02010009, LMGP02010010, LMGP02010099, LMGP02010311, LMGP02010378, LMGP02010415, LMGP02010440, LMGP02010462, LMGP02010491, LMGP02010524, LMGP02010544, LMGP02010569, LMGP02010770, LMGP02010797, LMGP02010824, LMGP02010843, LMGP02011040, LMGP02011204, LMGP03010098, LMGP03010117, LMGP03010168, LMGP03010206, LMGP03010251, LMGP03010280
726.49		•			•						$C_{40}H_{72}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010499, LMGP01010500, LMGP01010568, LMGP01010698, LMGP01011332, LMGP01011392, LMGP01011393, LMGP01011643, LMGP01011674, LMGP01011702, LMGP01011897, LMGP02010404, LMGP02010467, LMGP02010496, LMGP02010549, LMGP02010574, LMGP02010575, LMGP02010603, LMGP02010662, LMGP02010688, LMGP02010718, LMGP02010746, LMGP02010907, LMGP02010937, LMGP02011103
730.54					•						$C_{40}H_{76}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010494, LMGP01010496, LMGP01010682, LMGP01010684, LMGP01011330, LMGP01011390, LMGP01011414, LMGP01011440, LMGP01011526, LMGP01011555, LMGP01011597, LMGP01011616, LMGP01011835, LMGP02010403, LMGP02010465, LMGP02010494, LMGP02010528, LMGP02010546, LMGP02010572, LMGP02010601, LMGP02010627, LMGP02010641, LMGP02010660, LMGP02010800, LMGP02010846, LMGP02010875, LMGP02011072

735.55	•										$C_{40}H_{79}O_9P$	Glycerophosphoglycerol	LMGP04020044, LMGP04020072, LMGP04020092, LMGP04030012, LMGP04030034, LMGP04030058
740.52	•	•					•				$C_{41}H_{74}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01011356, LMGP01011418, LMGP01011445, LMGP01011446, LMGP01011645, LMGP01011676, LMGP01011704, LMGP01011898, LMGP02010096, LMGP02010111, LMGP02010112, LMGP02010421, LMGP02010532, LMGP02010643, LMGP02010691, LMGP02010749, LMGP02010909, LMGP02011104, LMGP02011174, LMGP02011190, LMGP02011197, LMGP02011203
744.56	•				•						$C_{41}H_{78}NO_8P$	Glycerophosphocholine or Glycerophosphoethanolamine	LMGP01010543, LMGP01011354, LMGP01011396, LMGP01011443, LMGP01011465, LMGP01011481, LMGP01011528, LMGP01011557, LMGP01011599, LMGP01011618, LMGP01011759, LMGP01011836, LMGP02010039, LMGP02010044, LMGP02010052, LMGP02010109, LMGP02010420, LMGP02010448, LMGP02010510, LMGP02010530, LMGP02010578, LMGP02010607, LMGP02010774, LMGP02010802, LMGP02010848, LMGP02010877, LMGP02011043, LMGP02011073, LMGP02011193, LMGP02011270
753.54			•					•	•	•	$C_{43}H_{77}O_8P$	Glycerophosophate	LMGP10010362, LMGP10010391, LMGP10010419, LMGP10010449, LMGP10010547, LMGP10010577, LMGP10010607, LMGP10010636, LMGP10010712, LMGP10010738, LMGP10010739, LMGP10010768, LMGP10010797, LMGP10010861, LMGP10010883,
795.53		•	•	•		•				•	$C_{41}H_{79}O_{12}P$	Glycerophosphoinositol	LMGP06020008, LMGP06020022, LMGP06030008, LMGP06030031, LMGP06030057
915.58								•	•	•	$C_{49}H_{87}O_{13}P$	Glycerophosphoinositol	LMGP06010332, LMGP06010361, LMGP06010389, LMGP06010419, LMGP06010517, LMGP06010547, LMGP06010577, LMGP06010606, LMGP06010682, LMGP06010708, LMGP06010709, LMGP06010738, LMGP06010767, LMGP06010831, LMGP06010853
931.56								•	•	•	$C_{51}H_{79}O_{13}P$	Glycerophosphoinositol	LMGP06010616, LMGP06010808
941.60			•	•		•				•	$C_{51}H_{89}O_{13}P$	Glycerophosphoinositol	LMGP06010524, LMGP06010585, LMGP06010613, LMGP06010642, LMGP06010689, LMGP06010717, LMGP06010747, LMGP06010776
945.57									•		$C_{45}H_{85}O_{18}P$	Glycerophosphoinositolglycan	LMGP15010002
973.59								•	•		$C_{47}H_{89}O_{18}P$	Glycerophosphoinositolglycan	LMGP15010003, LMGP15010014
1047.70			•	•		•					$C_{55}H_{102}N_2O_{16}$	Acidic glycosphingolipid	LMSP0601AA03

*measured monoisotopic m/z value of the protonated ion species, [#]obtained from LMSD with a mass tolerance of ±0.05 Da; [$]obtained from the main class category in LMSD. In general, flavonoids were excluded from the search results.

Table S3 – Most relevant lipid ion signals for the first 2 principle components of the PCA undertaken in this study (see Table S2 for accession number from LIPID MAPS structure database).

m/z	Putative lipid identification	Species detected in
607.50	Glycerophosphate	*S. aureus, S. epidermidis, S. pyogenes*
704.50	Glycerophosphocholine or Glycerophosphoethanolamine	*E. coli, K. pneumoniae*
730.50	Glycerophosphocholine or Glycerophosphoethanolamine	*C. jejuni, K. pneumoniae*
744.50	Glycerophosphocholine or Glycerophosphoethanolamine	*C. jejuni, K. pneumoniae*
915.50	Glycerophosphoinositol	*S. aureus, S. epidermidis, S. pyogenes*
931.50	Glycerophosphoinositol	*S. aureus, S. epidermidis, S. pyogenes*
941.50	Glycerophosphoinositol	*E. faecalis, E. hirae, L. brevis, S. pyogenes*
959.50	Glycerophosphoinositol	*E. faecalis, E. hirae, K. pneumoniae, S. epidermidis, S. pyogenes*

Chapter 4 – Rapid Multiplex Antimicrobial Resistance Profiling and Bacterial Identification by LAP-MALDI MS Biotyping

Research Article

Lellman SE, Adair L, Iyer S and Cramer R. Rapid Multiplex Antimicrobial Resistance Profiling and Bacterial Identification by LAP-MALDI MS Biotyping. Prepared as a Brief Communication for submission to Nature Medicine.

Author Contributions

Lellman SE – Design of experiment, undertaking investigation and analysis, drafting and editing the manuscript

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Rapid Multiplex Antimicrobial Resistance Profiling and Bacterial Identification by LAP-MALDI MS Biotyping

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Abstract

Rapid detection and correct characterisation of antimicrobial resistance result in earlier and more effective patient treatment. Infection-causing microorganisms often harbour multi-drug resistances, typically requiring multiple tests to identify these. Here, we present a multiplex assay using LAP-MALDI as the next-generation MALDI biotyping technology for accurate antibiotic resistance/susceptibility detection using a beta-lactam antibiotics panel with a short 3-h incubation time in a small <5-µL volume of bacterial culture solution. Bacteria with common resistance genes, including OXA-48, KPC-3 and VIM-1, as well as fully susceptible strains are easily classified. Beyond multi-drug testing, the same bacterial LAP-MALDI sample provides species-identifying lipid profiles (95-100% classification accuracy). Further analysis can be undertaken by including protein profiles and MS/MS protein sequencing, facilitated by LAP-MALDI's ability to generate multiply charged protein ions. MS/MS sequencing and high mass accuracy in measuring simultaneously multiple antibiotics, their products, and lipids provide new diagnostic possibilities in clinical microbiology that are less probability-based.

The requirement for effective microbial identification has become increasingly apparent by recent pandemics. Besides viral identification, effective bacterial and other microbial identification is much needed and can improve patient outcome and reduce healthcare costs. Treatment of bacterial infections typically includes a bacterium-dependent course of antibiotics. However, the use of antibiotics has contributed to the emergence of antibiotic-resistant strains of bacteria, with extreme cases meaning the last-line-of-defence antibiotics are no longer effective(1, 2). Antibiotic resistance is estimated to add €1.6 billion to hospital costs within Europe(3), and to be the leading cause of death in humans globally(4), therefore highlighting the need for early and effective microbial identification and detection of antibiotic resistance/susceptibility of clinical infections.

A major cause of resistance to β-lactam antibiotics such as carbapenems is the production of β-lactamases which hydrolyse the β-lactam ring(5). Out of the six leading pathogens responsible for death associated with resistance, four commonly possess carbapenem resistance(4). The use of carbapenems has increased due to the increased resistance of Enterobacteriaceae to cephalosporin antibiotics, another class of beta-lactams(6). Carbapenems are considered as a last-resort antibiotic due to their relative resistance to hydrolysis by most β -lactamases(6, 7). In turn, increased carbapenem use has led to the emergence of carbapenemases, creating a snowball effect leading to multi-drug resistant organisms (MDRO).

Current clinical methods of resistance detection include phenotypic tests, such as disc diffusion assays and nucleic acid tests, detecting genetic components known to cause β-lactam resistance. Phenotypic assays typically take 1-3 days following bacterial culture(8) and often result in false positives(9). Nucleic acid tests such as PCR are more rapid but still

incur high reagent costs, and the genetic target of interest must be known prior to testing. MALDI-TOF MS assays for cephalosporinase and carbapenemase activity detection have been developed based on the antibiotics' mass shift due to hydrolysis of the β -lactam ring but only against a single benchmark antibiotic(10, 11). More recently, machine learning has been applied to MALDI-TOF MS profiles for resistance prediction, with the ability to discriminate between resistance mechanisms(12).

LAP-MALDI MS has been developed and optimised within the last decade(13) and has shown its success in identifying clinically important bacteria(14), as well as in veterinary diagnostics(15). Both applications have utilised species/class-specific lipid and protein profiles for identification/classification. Here, we extend mass spectral profiling with multiplex antibiotic resistance detection, all in one assay. This assay exploits the easiness of acquiring lipid profiles and individual antibiotic ion signals at high sensitivity and mass accuracy using LAP-MALDI Q-TOF MS analysis of a single sample within seconds. LAP-MALDI MS can be further applied to bacterial protein sequencing. Thus, LAP-MALDI MS can provide both bacterial identification and multiplex antibiotic resistance testing, the latter directly from discrete mass spectral signals rather than relying on prediction models built by supervised machine learning.

To demonstrate the ability of LAP-MALDI MS profiling in identifying a wide range of bacteria, Gram-positive *S. aureus* and Gram-negative *E. coli* as well as members of the same genus such as *K. pneumoniae* and *K. aerogenes* were included in this study. All eight species included are clinically important and have the potential to exhibit antimicrobial resistance.

Bacteria were subjected to a simple ethanol extraction protocol(14), which has been further simplified by removing the additional application of formic acid. LAP-MALDI MS analysis of each bacterium produced a unique lipid profile in the m/z region of 600-1000 within 30 seconds. A clear visual distinction can be observed between the MS profiles of Gram-positive and Gram-negative bacteria (Figure 1A).

Lipid profiles are easily obtained for bacteria using mass spectrometry(16). Lipids are key structural components of bacterial cell walls, explaining why there is a clear difference in the lipid profiles between Gram-negative and Gram-positive bacteria as observed here. Lipid identification using MALDI-based techniques is typically excluded from biotyping axial-TOF instruments due to high matrix ion interference and relatively low mass accuracy, thus focusing simply on proteomic profiles. An advantage of LAP-MALDI on Q-TOF instrumentation is the presence of fewer of these interfering peaks and higher mass accuracy/resolution, providing high quality lipidomic MS profiles as well as the opportunity to identify individual lipids by accurate mass measurement and MS/MS analysis(15, 17).

After MS ion signals were processed and binned (into 1-mass-unit bins), the data was linearised based upon 20 Principal Component Analysis (PCA) dimensions. Linear Discriminant Analysis (LDA) was then performed with 5 dimensions, using a mass range of 660-1000 units and excluding matrix ion cluster signals at m/z 656 and 1078. Visualisation of the LDA in 3 dimensions shows clear separation and clustering of individual species, as well

as a clear separation between Gram-positive (Figure 1B; upper right back octant) and Gram-negative species (Figure 1B; other octants). Based on these profiles of mainly lipids, cross-validation shows a classification accuracy of 100%.

The high mass accuracy/resolution of LAP-MALDI Q-TOF MS is particularly advantageous in the low m/z range where smaller antibiotics and their products are recorded. Consequently, bacterial identification by lipid profiling was extended by analysing individual antibiotic ion signals using the same mass spectra as already acquired. For this combined analysis, single MS data acquisitions from the same sample can be used, incubating <5-µL of harvested bacterial culture solution with multiple antibiotics for only three h. In this case, two *E. coli* and *K. pneumoniae* strains with no known resistance mechanism and 5 strains with resistance genes, conferring resistance to some, if not all, beta-lactam antibiotics, were analysed. β-lactam antibiotics from three different classes (ampicillin, cephalexin, meropenem) were selected to create a clinically relevant antibiotic test mixture.

Samples were prepared using simple 3-h antibiotic incubation, followed by a rapid one-pot, single-step ethanol extraction, which simultaneously inactivates bacteria(18) and is therefore particularly beneficial for analysing highly pathogenic (biosafety) level-three organisms. A simple centrifugation step collects insoluble material, and the supernatants are spotted 1:1 with the LAP-MALDI matrix for MS analysis. Although data was manually acquired, there is scope for automation and further reduction in analysis time(19).

LAP-MALDI mass spectra of the incubation assay possess both antibiotic-related peaks and lipid profiles from the bacterium (Figure 1C) with the lipids being detected as previously, roughly around m/z 600-1200 (Figure 1D). Antibiotic-related peaks can be observed in the lower m/z range of the mass spectrum (Figure 1E) with high mass accuracy and resolution

(Supplementary Data Table 1). Due to PBS, intact doubly sodiated antibiotics (ampicillin [M-H+2Na]$^+$: m/z 394, cefalexin [M-H+2Na]$^+$: m/z 392, meropenem [M-H+2Na]$^+$: m/z 428) are the main ion signals for strains that are antibiotic-sensitive (Figure 1E). For the antibiotic-resistant bacteria and in agreement with previous LAP-MALDI studies(15), the doubly sodiated ion species of the hydrolysed decarboxylated antibiotics are detected (ampicillin [M+H_2O-CO_2-H+2Na]$^+$: m/z 368, cefalexin [M+H_2O-CO_2-H+2Na]$^+$: m/z 366, meropenem [M+H_2O-CO_2-H+2Na]$^+$: m/z 402;Figure 1E). Besides accurate mass measurements (Supplementary Data Table 1) and MS/MS analysis (Supplementary Data Figure 1 for meropenem), the assignments of these ion signals were also confirmed by solely analysing the antibiotics and incubating them with penicillinase-spiked susceptible strains, which provided the same antibiotic product ions as those obtained by incubation with the resistant strains (see Supplementary Data Figure 2).

LDA as performed previously shows clear discrimination between *E. coli* and *K. pneumoniae* and between the antibiotics-susceptible and resistant strains (Figure 1F). The greater separation between the sensitive and resistant strains than between the species is a clear indication of the strong influence of antibiotic/matrix clusters in the m/z range used, e.g. seen at m/z 814, 816, 850 (for susceptible strains) and m/z 788, 790, 824 (for resistant strains), and provides an additional option to develop a different dual assay for bacterial AMR testing and species identification, entirely based on profiling. The LDA loading plot (Supplementary Data Figure 3) reveals two main peaks responsible for separation; m/z 790.14 in resistant strains, and m/z 816.13 in susceptible strains, both identified as antibiotic/matrix cluster ions by MS/MS and accurate mass measurement.

LDA analysis of all MS profiles used for Figure 1 and 2 without the identified cluster ion signals of the antibiotics and their products (Figure 1G) led to a bacterial identification accuracy of 95%. This mixture analysis and the close species-specific clustering of the two sample preparations (with and without 3-h antibiotic incubation) demonstrates the robustness of the assay for bacterial identification with respect to variations in timelines and the addition of antibiotics.

To assess the level of resistance exhibited by the bacteria, a ratio (resistance) score was calculated between the intact antibiotic and its hydrolysed decarboxylated product ion signals (Supplementary Data Table 1 for m/z values), based on the peak area of the doubly sodiated ion signals as these were the most intense.

Calculated average resistance scores are in the range of 0-78 (Figure 2A). The two susceptible strains exhibit resistance scores between 0 and 0.35 for all antibiotics. Apart from *K. pneumoniae* (OXA-48), all resistant strains show resistance scores of >15, i.e. approximately a 40-fold difference to the susceptible strain scores. Importantly, *K. pneumoniae* (OXA-48) has a resistance score of 17 against ampicillin but approximately 0 for cefalexin and meropenem. OXA-48 is a class-D lactamase gene resulting in strong resistance to penicillins, and weak hydrolysis activity against cephalosporins and carbapanems(20, 21), as confirmed by this data with a resistance score of 17 for the OXA-48 strain against ampicillin, and close to 0 for cefalexin and meropenem.

Considering the clear antibiotic-specific separation between lactamase-active and inactive strains, a scoring system could be devised that provides rapid clinically relevant information on the bacteria's AMR status, providing effective guidance on the use of antibiotics. As there is a clear 'dead-zone' at the score range 1-10 with 50% margins at the limits where no

bacterium in this study produced a score (Figure 2B), a traffic-light system could be employed with effective cut-off threshold levels.

Furthermore, current profiling analysis in clinical MALDI MS biotyping could be fully abandoned with LAP-MALDI by applying protein sequencing using MS/MS analysis (Figure 2C) and protein sequence database searching (Figure 2D) in addition to lipidomic and proteomic profiles. Identification of (bacterial) species by sequencing using MS/MS data will be more reliable than by simply MS profiling analysis using class prediction models, adding additional specificity and flexibility to the mass spectral workflows in clinical microbiology.

In summary, LAP-MALDI MS analysis of clinically important and diverse bacterial strains (antibiotic-susceptible and resistant) has been shown to classify bacteria with 95-100% accuracy and their AMR status with 100% accuracy in a multi-drug assay (Figure 2E). By employing Q-TOF instrumentation, the assay provides high mass accuracy and resolution, and thus the potential to include many more antibiotics. Moreover, lipid profiling can be substituted or supplemented by protein sequencing, exploiting LAP-MALDI's ability to generate multiply charged protein ions, and therefore high-quality MS/MS spectra on high-performing hybrid mass analysers. The new possibilities LAP-MALDI offers in clinical microbiology range from the current protein profiling, new lipid and combined lipid/protein profiling to highly accurate mass measurements of multiple antibiotics, lipids and proteins as well as bacterial protein sequencing from small volumes of bacterial culture (with short multi-drug incubation times). As a consequence, new MALDI assays can be developed that can go beyond singular antibiotic testing or bacterial identification to rapid multiplex bacterial characterisation that includes both bacterial identification and multi-drug resistance testing with high accuracy.

Materials

First generation NCTC bacterial strains were purchased as freeze-dried discs from Pro-Lab Diagnostics (Wirral, UK). These included *Escherichia coli* (NCTC 13386), *Enterobacter cloacae* (NCTC 13380), *Klebsiella pneumoniae* (NCTC9633), *Pseudomonas aeruginosa* (NCTC 12903), *Staphylococcus aureus* (NCTC 6571), *Klebsiella aerogenes* (NCTC 9528), *Staphylococcus epidermidis* (NCTC 13360) and *Acinetobacter baumanii* (NCTC 12156). Strains with antibiotic resistance and their respective resistance genes were *E. coli* (NCTC 13438, IMP-1) and four *K. pneumoniae strains* (NCTC 13440, VIM-1; NCTC 13443, NDM-1; NCTC 13438, KPC-3 and NCTC 13442, OXA-48).

Dehydrated nutrient agar culture medium was purchased from Oxoid (Thermo Fisher, Basingstoke, UK). Antibiotic compounds, penicillinase and matrix components were purchased from Sigma Aldrich (Gillingham, UK). HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK).

Growth and Preparation of Bacteria

Bacteria were revived from freeze-dried discs according to the supplier's instructions and stored in 70% glycerol at -80°C until required. For propagation, a loopful of the glycerol stock was streaked onto a solid nutrient agar plate and incubated at 37°C for 24 h. Approximately 5 µL of biological material was harvested, using the pointed end of a disposable bacterial loop, and resuspended in 60 µL of 1XPBS (phosphate-buffered saline). Ethanol was added to a final concentration of 70% (v/v), and samples were centrifuged for 2 min at 13,000 rpm. The supernatant was used for the analyses without antibiotic incubation.

Antibiotic Assay

A volume of 20 µL of an aqueous antibiotic mixture containing ampicillin (4 mg/mL), meropenem (4 mg/mL) and cefalexin (4 mg/mL) was added to each bacterial suspension prior to adding ethanol. The suspensions were then incubated at 37°C for 3 h. Following incubation, ethanol was added to a final concentration of 70% (v/v), and samples were centrifuged for 2 min at 13,000 rpm. The supernatant was used for analysis. Penicillinase (1 µL of 1 mg/mL final concentration in H_2O) was spiked into susceptible strains as a control, followed by incubation at 37°C for 3 h.

Protein Extraction

A small volume of biological material was harvested from incubated solid media plates as above, and added to 60µL 1X PBS. 20µL of antibiotic mixture was added and the suspensions were incubated at 37°C for 3 h. A volume of 4µL 100% TCA was added to suspensions and left on ice to precipitate for 30 min. Samples were centrifuged for 2 min at 13,000 rpm and the supernatant removed. The pellets were washed in acetone and centrifuged at 13,000 rpm for 2 min. The supernatant removed and the resultant pellet resuspended in 30 µL 0.1% TFA, followed by clean-up using a C18 ZipTip. The final elution volume was 5µL.

Replicates

Where samples were only subjected to ethanol extraction, 3 biological replicates were prepared, and 3 technical replicates were analysed from each biological replicate, totalling 9 samples per bacterium. For the samples with antibiotic incubation, 7 biological replicates

were prepared, each analysed as a single technical replicate. The penicillinase assay was performed with 3 biological replicates, each analysed as a single technical replicate.

LAP-MALDI MS Analysis

A CHCA-based liquid support matrix (LSM) was used for all analyses, composed of 25-mg/mL CHCA in acetonitrile:water (70:30%, v/v), with subsequently added propylene glycol at 70% volume. This LSM was thoroughly vortexed and mixed 1:1 with the bacterial extract on a stainless-steel MALDI sample plate, producing a 1-µL liquid droplet for each MALDI sample.

A Synapt G2-Si instrument (Waters, Wilmslow, UK) with an in-house built AP-MALDI source was used for data acquisition. The standard electrospray ionisation source was modified with a cone adaptor connected to a heated inlet capillary, which was heated by a resistance wire embedded in thermally conductive but electrically non-conductive cement and connected to an external power supply. The inlet capillary was directed at a vertically mounted MALDI sample plate, magnetically attached to an XYZ stage. A 343-nm UV laser was operated at 30Hz and its beam was focused onto the MALDI sample droplet, providing around 10-20 µJ per pulse for desorption.

All data were acquired in mobility TOF and sensitivity mode. Calibration was performed using sodium iodide (2 µg/µL in isopropanol). The source was operated in positive ion mode at a potential of approximately 3 kV (between MALDI plate and capillary inlet), an outside temperature of the capillary assembly of approximately 300°C, and 180 L/h N_2 counter-gas flow. The instrument's desolvation temperature was set to 350°C, the desolvation gas flow was set to 600 L/h, and the purge gas flow was set to 600 mL/h. For MS/MS experiments, the trap collision energy was set to 20V.

Data was acquired using MassLynx 4.2® (Waters), and each sample was analysed for 30 seconds at 1 scan per second. For data presentation and analysis, spectra from all scans were summed.

Linear Discriminant Analysis (LDA)

Statistical analysis was performed using AMX Abstract Model Builder® (Waters). Spectra from all scans were combined for each data file, one spectrum per sample included in the model. For model building the AMX Abstract Model Builder® binned the data every 1 mass unit, followed by LDA in the m/z range of 660-1000 with 20 PCA dimensions for linearising the data, and 5 LDA dimensions. Background subtraction and normalisation pre-processing were selected. Cross-validation was performed using the built-in '20% out' function, where outliers were based on 5 standard deviations. Outliers only occurred in the combined lipid profile / antibiotic-resistant analysis and would be samples to be re-analysed in a clinical laboratory setting.

Calculation of Resistance Scores

Ion signal (area) intensity values were obtained by converting continuum data files to centroid data using 'Automatic Peak Detection with Peak Filter' under the 'Process' menu in MassLynx 4.2® with background subtraction, Savitzky Golay smoothing and area integration as applied to the calibration. For the resistance score, the ion signal intensity of the doubly sodiated hydrolysed decarboxylated antibiotic was divided by the ion signal intensity of the doubly sodiated intact antibiotic. Three biological replicates were used for each strain and the overall score was based on the mean intensity values.

Identification of Proteins

The obtained MS/MS spectra were opened in MASCOT Distiller (Version 2.8.3; Matrix Science, London, UK) for deconvolution. Automated peak picking settings were used, including a minimum signal to noise (S/N) of 5, with baseline correction. The m/z range used was 100-2000 for MS and MS/MS peak picking. The peak list was exported as singly charged equivalents of the multiply charged fragment ions detected. A MASCOT search was performed using no fixed or variable modifications, with 'NoCleave' selected as the enzyme. A parent ion tolerance of 50 ppm was used, and a fragment ion tolerance of 0.2 Da.

Figures

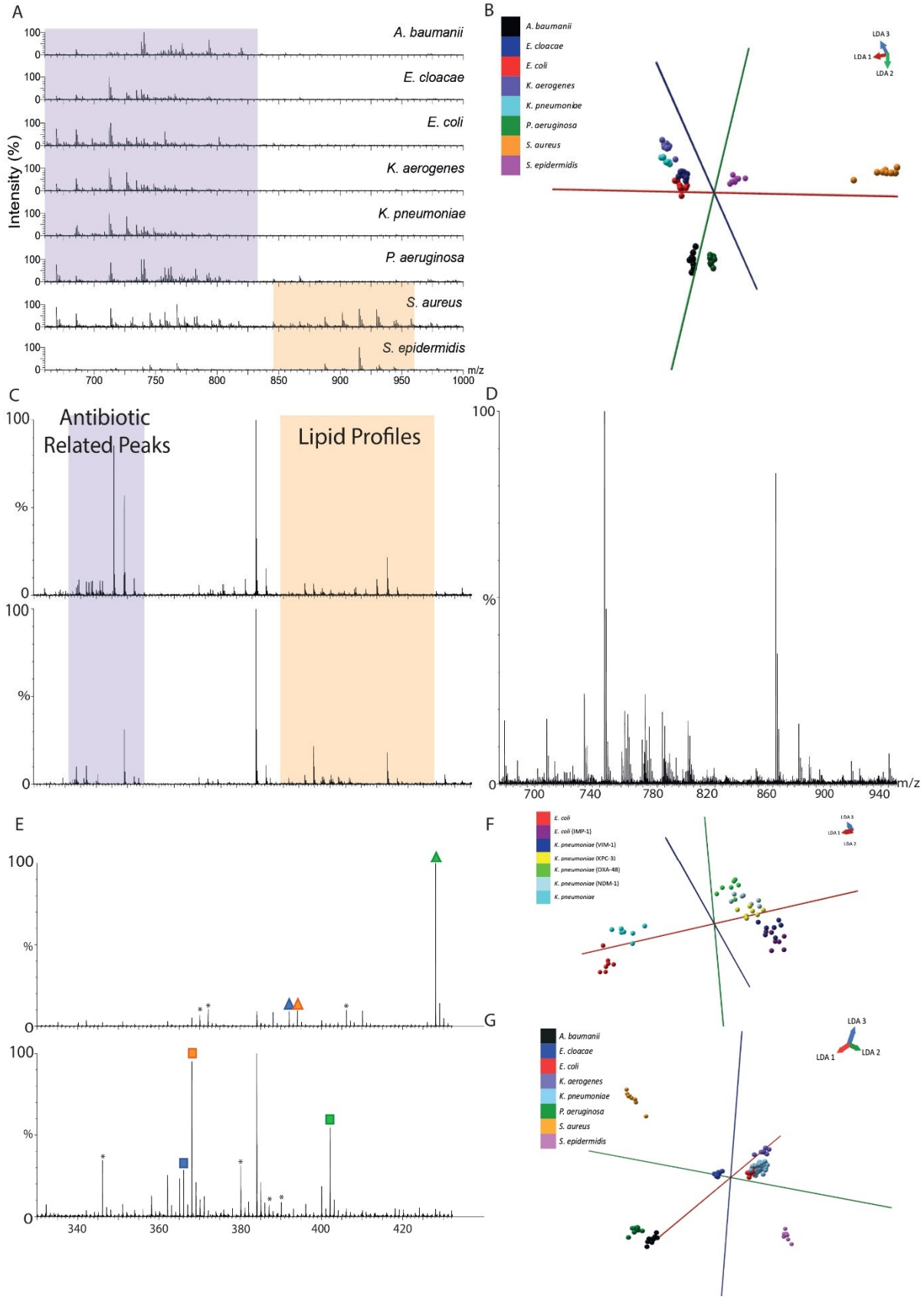
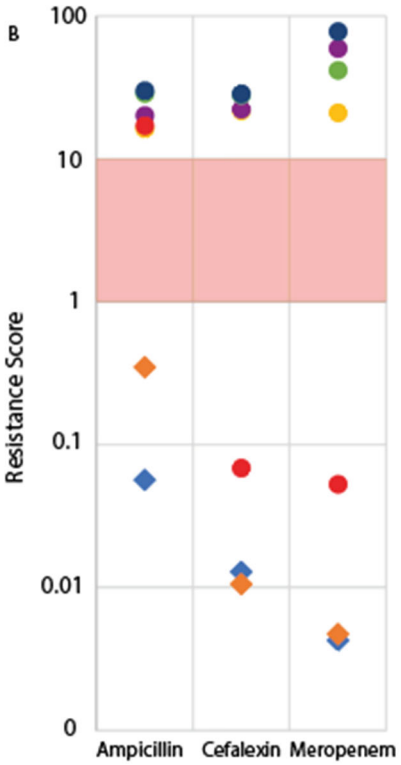

A
A. baumanii
E. cloacae
E. coli
K. aerogenes
K. pneumoniae
P. aeruginosa
S. aureus
S. epidermidis
Intensity (%)
m/z
B
C
Antibiotic Related Peaks
Lipid Profiles
D
E
F
G

Figure 1 – Acquisition of LAP-MALDI MS spectra detecting hydrolysis of antibiotics and lipid profiles in the same spectrum (C), focusing on the lipid profile region of *E. coli* (D). The detection of antibiotic resistance (E) is determined by the detection of the intact antibiotic peaks (▲) and hydrolysed decarboxylated peaks (■), additional antibiotic related peaks are indicated by (*). Visualisation of the LDA of lipid profiles for each species used in the antibiotic assay (F) shows clear distinction of resistance and sensitive strains. Species identification of lipid profiles with and without antibiotic incubation is visualised in (G).

A

	Ampicillin	Cefalexin	Meropenem
E. coli ◆	0.06	0.01	0.00
K. pneumoniae ◆	0.35	0.01	0.00
K. pneumoniae (VIM-1) ●	16.32	21.73	21.07
K. pneumoniae (NDM-1) ●	20.11	22.40	59.45
E. coli (IMP-1) ●	28.76	28.09	41.79
K. pneumoniae (KPC-3) ●	30.02	28.63	77.88
K. pneumoniae (OXA-48) ●	17.07	0.07	0.05

B



C

MULTISPECIES: DNA-binding protein HU-alpha [Enterobacterales]

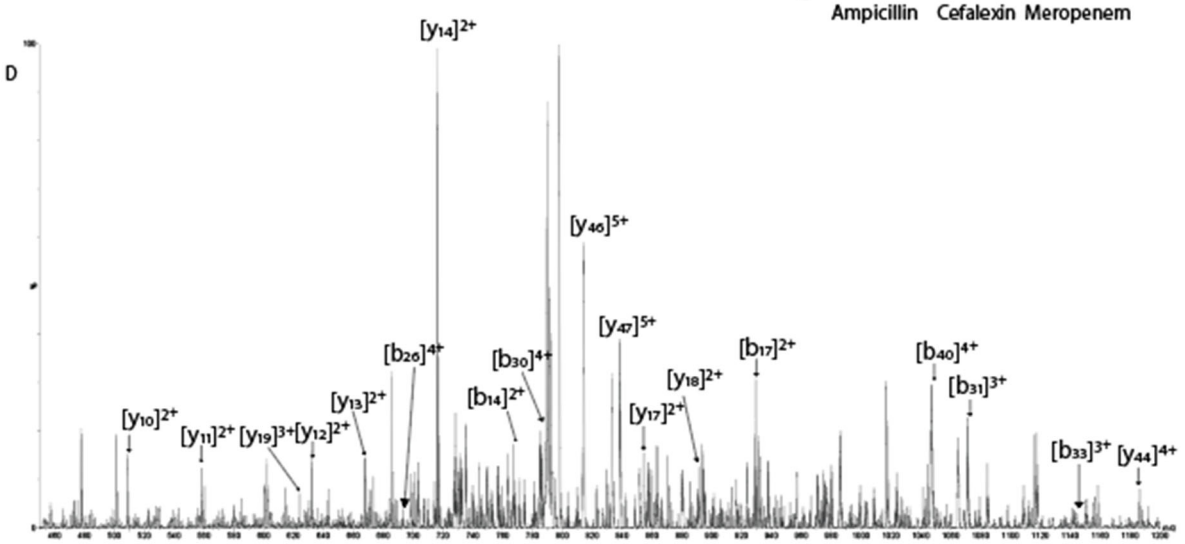
Database: NCBIprot
Score: 55
Monoisotopic mass (M_r): 9471
Calculated pI: 9.57
Taxonomy: Enterobacterales

Protein sequence coverage: 100%

Matched peptides shown in ***bold red***.

1 MNKTQLIDVI ADKADLSKAQ AKAALESTLA AITESLKEGD AVQLVGFGTF
51 KVNHRAERTG RNPQTGKEIK IAAANVPAFV SGKALKDAVK

D



E

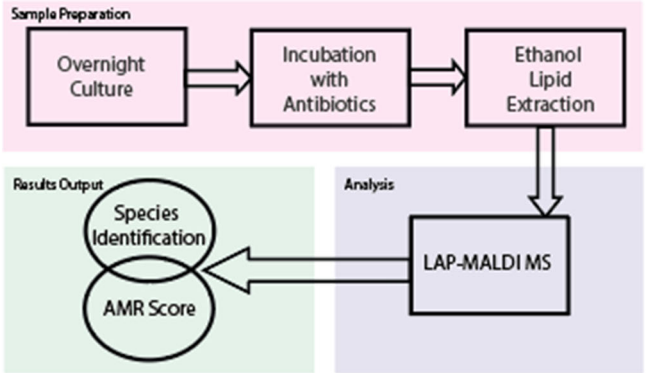


Figure 2 – Scores of resistance calculated from a ratio of the doubly sodiated intact antibiotic in comparison to the doubly sodiated hydrolysed decarboxylated antibiotic (A) displayed on a logarithmic scale (B). Proteomic identification of biomarkers in a spectrum (C) from LAP-MALDI MS/MS (D) allows for higher identifying power for species identification. Antibiotic resistance detection and species identification can be performed using LAP-MALDI MS via a simple, rapid workflow (E).

1. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. The Lancet Infectious Diseases. 2019;19(1):56-66.
2. Raoult D, Leone M, Roussel Y, Rolain J-M. Attributable deaths caused by infections with antibiotic-resistant bacteria in France. The Lancet Infectious Diseases. 2019;19(2):128-9.
3. Ahmad M, Khan AU. Global economic impact of antibiotic resistance: A review. Journal of Global Antimicrobial Resistance. 2019;19:313-6.
4. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022;399(10325):629-55.
5. Babic M, Hujer AM, Bonomo RA. What's new in antibiotic resistance? Focus on beta-lactamases. Drug Resistance Updates. 2006;9(3):142-56.
6. Hawkey PM, Livermore DM. Carbapenem antibiotics for serious infections. BMJ : British Medical Journal. 2012;344:e3236.
7. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: past, present, and future. Antimicrobial Agents and Chemotherapy. 2011;55(11):4943-60.
8. van Belkum A, Burnham C-AD, Rossen JWA, Mallard F, Rochas O, Dunne WM. Innovative and rapid antimicrobial susceptibility testing systems. Nature Reviews Microbiology. 2020;18(5):299-311.
9. Carvalhaes CG, Picão RC, Nicoletti AG, Xavier DE, Gales AC. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in Klebsiella pneumoniae: be aware of false positive results. Journal of Antimicrobial Chemotherapy. 2009;65(2):249-51.
10. Anantharajah A, Tossens B, Olive N, Kabamba-Mukadi B, Rodriguez-Villalobos H, Verroken A. Performance Evaluation of the MBT STAR®-Carba IVD Assay for the Detection of Carbapenemases With MALDI-TOF MS. Frontiers in Microbiology. 2019;10.
11. Ota Y, Furuhashi K, Hirai N, Ishikawa J, Nagura O, Yamanaka K, et al. Evaluation of MBT STAR-Cepha and MBT STAR-Carba kits for the detection of extended-spectrum β-lactamases and carbapenemase producing microorganisms using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Journal of Microbiological Methods. 2021;183:106166.
12. Weis C, Cuénod A, Rieck B, Dubuis O, Graf S, Lang C, et al. Direct antimicrobial resistance prediction from clinical MALDI-TOF mass spectra using machine learning. Nature Medicine. 2022;28(1):164-74.
13. Cramer R, Pirkl A, Hillenkamp F, Dreisewerd K. Liquid AP-UV-MALDI Enables Stable Ion Yields of Multiply Charged Peptide and Protein Ions for Sensitive Analysis by Mass Spectrometry. Angewandte Chemie International Edition. 2013;52(8):2364-7.
14. Lellman SE, Cramer R. Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Clinical Chemistry and Laboratory Medicine. 2020;58(6):930-8.
15. Piras C, Hale OJ, Reynolds CK, Jones AK, Taylor N, Morris M, et al. LAP-MALDI MS coupled with machine learning: an ambient mass spectrometry approach for high-throughput diagnostics. Chemical Science. 2022;13(6):1746-58.
16. Shu X, Li Y, Liang M, Yang B, Liu C, Wang Y, et al. Rapid lipid profiling of bacteria by online MALDI-TOF mass spectrometry. International Journal of Mass Spectrometry. 2012;321-322:71-6.
17. Hale OJ, Cramer R. Collision-induced dissociation of doubly-charged barium-cationized lipids generated from liquid samples by atmospheric pressure matrix-assisted laser desorption/ionization provides structurally diagnostic product ions. Analytical and Bioanalytical Chemistry. 2018;410(5):1435-44.
18. Freiwald A, Sauer S. Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols. 2009;4(5):732-42.

19. Krenkel H, Brown J, Richardson K, Hoyes E, Morris M, Cramer R. Ultrahigh-Throughput Sample Analysis Using Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Analytical Chemistry. 2022;94(10):4141-5.
20. Poirel L, Potron A, Nordmann P. OXA-48-like carbapenemases: the phantom menace. Journal of Antimicrobial Chemotherapy. 2012;67(7):1597-606.
21. Stewart A, Harris P, Henderson A, Paterson D. Treatment of Infections by OXA-48-Producing Enterobacteriaceae. Antimicrobial Agents and Chemotherapy. 2018;62(11).
22. Ryumin P, Brown J, Morris M, Cramer R. Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS. Methods. 2016;104:11-20.

Supplementary Information

Rapid Multiplex Antimicrobial Resistance Profiling and Bacterial Identification by LAP-MALDI MS Biotyping

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Supplementary Information Table 1A – Putative lipid identification in *K. pneumoniae* based on accurate mass measurements using LIPIDMAPs database (https://lipidmaps.org/databases/lmsd/)

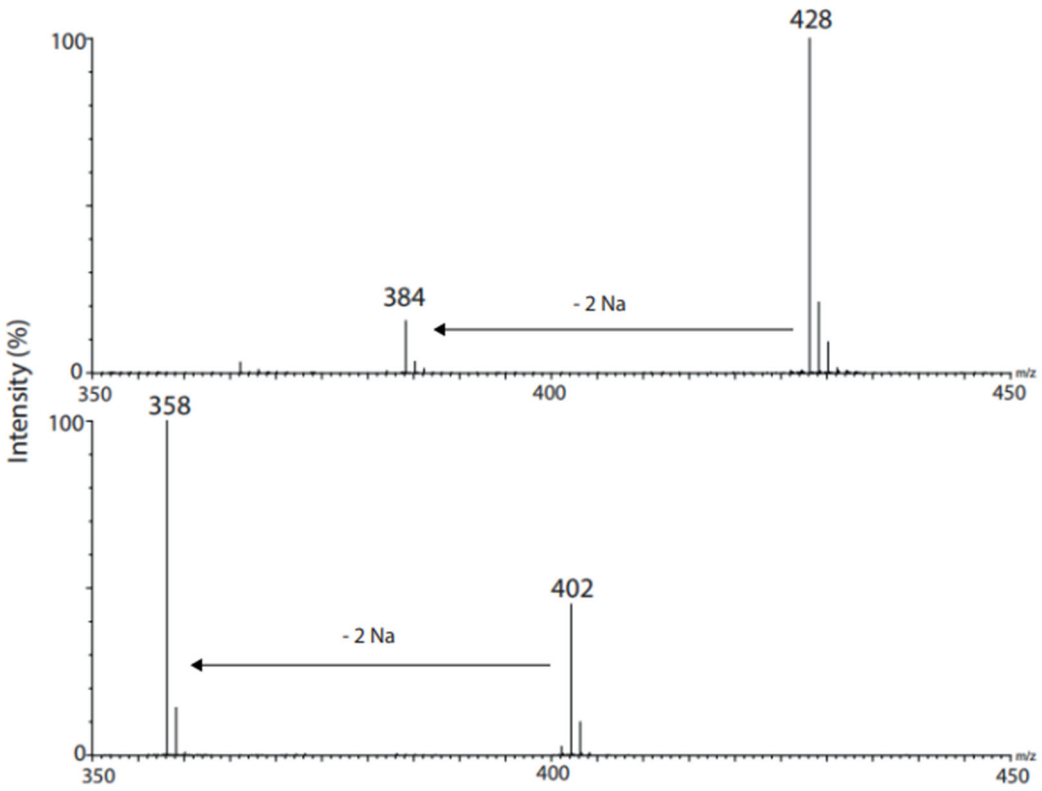
Input Mass	Matched Mass	Delta ppm	Name	Formula	Ion
669.4802	669.4854	4.1823	PA 35:2	$C_{38}H_{71}O_8P$	$[M+H-H_2O]^+$
	669.483	-3.5848	PA O-33:1	$C_{36}H_{71}O_7PNa$	$[M+Na]^+$
	669.4813	1.6431	LPS O-27:1;O	$C_{33}H_{66}NO_9P$	$[M+NH_4]^+$
685.4338	685.4317	3.0637	CerPE 32:2;O3	$C_{34}H_{67}N_2O_7PK$	$[M+K]^+$
686.4625	686.4578	8.8467	HexCer 30:2;O2	$C_{36}H_{67}NO_8Na_2$	$[M+2Na-H]^+$
712.4839	712.4888	-6.8773	PC 29:1	$C_{37}H_{72}NO_8PNa$	$[M+Na]^+$
	712.4888	-6.8773	PE 32:1	$C_{37}H_{72}NO_8PNa$	$[M+Na]^+$
713.4915	713.4864	7.1479	PE 33:5	$C_{38}H_{66}NO_8P$	$[M+NH_4]^+$
	713.4882	4.6252	PA O-35:1	$C_{38}H_{75}O_7PK$	$[M+K]^+$
726.4995	726.5044	-6.7446	PC 30:1	$C_{38}H_{74}NO_8PNa$	$[M+Na]^+$
	726.5044	-6.7446	PE 33:1	$C_{38}H_{74}NO_8PNa$	$[M+Na]^+$
734.4687	734.4755	-9.2583	PE 36:7	$C_{41}H_{68}NO_8P$	$[M+H]^+$
	734.4731	5.9907	PC 31:4	$C_{39}H_{70}NO_8PNa$	$[M+Na]^+$
	734.4731	5.9907	PE 34:4	$C_{39}H_{70}NO_8PNa$	$[M+Na]^+$
	734.4755	9.2583	PA 38:8	$C_{41}H_{65}O_8P$	$[M+NH_4]^+$
	734.4707	2.7230	PC 29:1	$C_{37}H_{72}NO_8PNa_2$	$[M+2Na-H]^+$
	734.4707	2.7230	PE 32:1	$C_{37}H_{72}NO_8PNa_2$	$[M+2Na-H]^+$
740.5129	740.5201	9.7228	PC 31:1	$C_{39}H_{76}NO_8PNa$	$[M+Na]^+$
	740.5201	9.7228	PE 34:1	$C_{39}H_{76}NO_8PNa$	$[M+Na]^+$
743.4821	743.4858	4.9766	PG 34:4	$C_{40}H_{71}O_{10}P$	$[M+H]^+$
	743.4834	1.7485	PG 32:1	$C_{38}H_{73}O_{10}PNa$	$[M+Na]^+$
748.483	748.4759	9.4859	PC 30:3;O3	$C_{38}H_{70}NO_{11}P$	$[M+H]^+$
	748.4888	7.7489	PC 32:4	$C_{40}H_{70}NO_8PNa$	$[M+Na]^+$
	748.4888	7.7489	PE 35:4	$C_{40}H_{70}NO_8PNa$	$[M+Na]^+$
	748.4864	4.5425	PC 30:1	$C_{38}H_{74}NO_8PNa_2$	$[M+2Na-H]^+$
	748.4864	4.5425	PE 33:1	$C_{38}H_{74}NO_8PNa_2$	$[M+2Na-H]^+$
754.5206	754.523	3.1808	HexCer 34:1;O3	$C_{40}H_{77}NO_9K$	$[M+K]^+$
	754.5147	7.8196	PC O-32:2	$C_{40}H_{78}NO_7PK$	$[M+K]^+$
	754.5147	7.8196	PE O-35:2	$C_{40}H_{78}NO_7PK$	$[M+K]^+$
765.4647	765.4701	7.0545	PG 36:7	$C_{42}H_{69}O_{10}P$	$[M+H]^+$
	765.4677	3.9192	PG 34:4	$C_{40}H_{71}O_{10}PNa$	$[M+Na]^+$
	765.4653	0.7838	PG 32:1	$C_{38}H_{73}O_{10}PNa_2$	$[M+2Na-H]^+$
779.4752	779.4705	0.0047	PI 30:2	$C_{39}H_{71}O_{13}P$	$[M+H]^+$
	779.4809	0.0057	PG 33:1	$C_{39}H_{75}O_{10}PNa_2$	$[M+2Na-H]^+$
793.4902	793.4862	5.0410	PI 31:2	$C_{40}H_{73}O_{13}P$	$[M+H]^+$
	793.4966	8.0656	PG 34:1	$C_{40}H_{77}O_{10}PNa_2$	$[M+2Na-H]^+$
945.4933	945.489	4.5479	PI 40:8	$C_{49}H_{79}O_{13}PK$	$[M+K]^+$

Supplementary Information Table 1B – Putative lipid identification in *S. aureus* based on accurate mass measurements using LIPIDMAPs database (https://lipidmaps.org/databases/lmsd/)

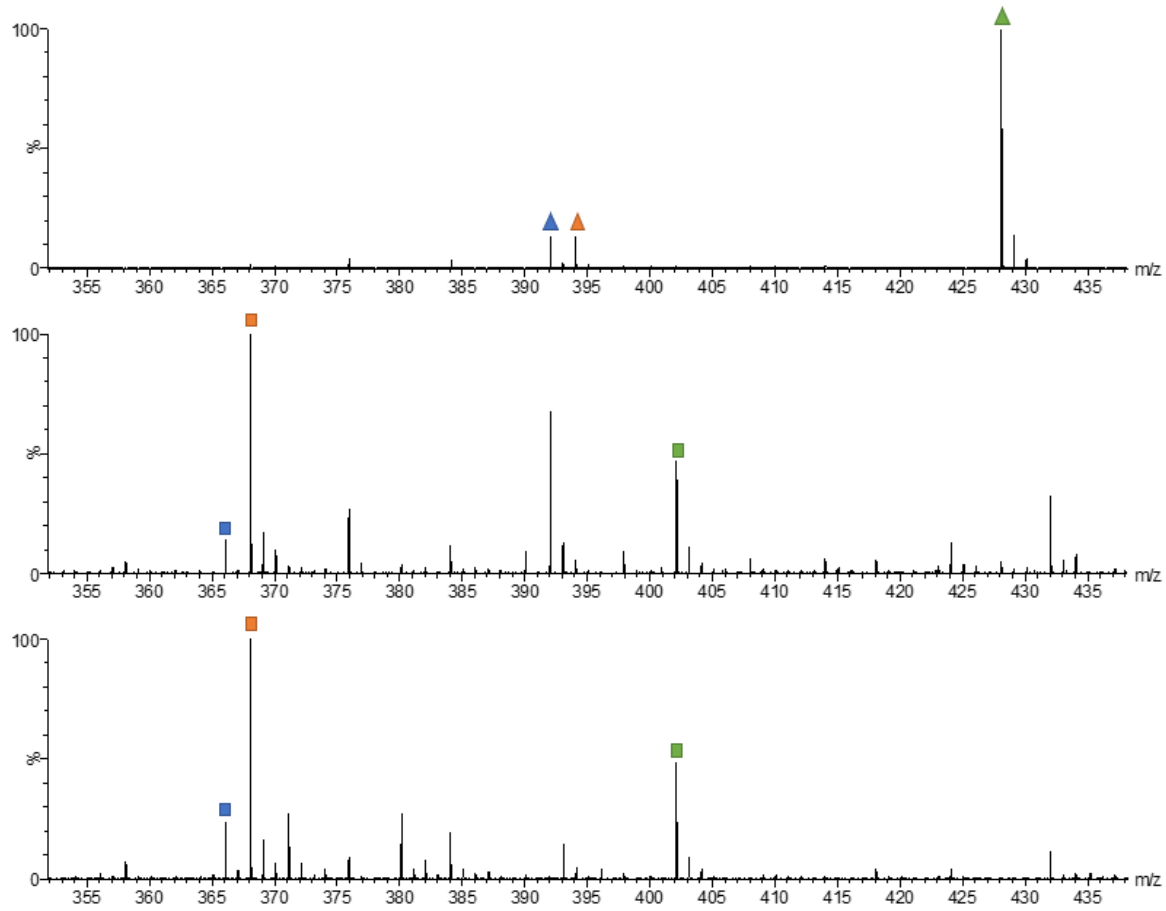
Observed Mass	Theoretical Mass	Delta ppm	Name	Formula	Ion
717.4658	717.4701	5.9933	PG 32:3	$C_{38}H_{69}O_{10}P$	$[M+H]^+$
	717.4677	2.6482	PG 30:0	$C_{36}H_{71}O_{10}PNa$	$[M+Na]^+$
739.4506	739.4545	5.2742	PG 34:6	$C_{40}H_{67}O_{10}P$	$[M+H]^+$
	739.4521	2.0285	PG 32:3	$C_{38}H_{69}O_{10}PNa$	$[M+Na]^+$
	739.4496	-1.3524	PG 30:0	$C_{36}H_{71}O_{10}PNa_2$	$[M+2Na-H]^+$
745.4979	745.5014	4.6948	PG 34:3	$C_{40}H_{73}O_{10}P$	$[M+H]^+$
	745.499	1.4755	PG 32:0	$C_{38}H_{75}O_{10}PNa$	$[M+Na]^+$
767.4802	767.4858	7.2965	PG 36:6	$C_{42}H_{71}O_{10}P$	$[M+H]^+$
	767.4834	4.1695	PG 34:3	$C_{40}H_{73}O_{10}PNa$	$[M+Na]^+$
	767.4809	0.9121	PG 32:0	$C_{38}H_{75}O_{10}PNa_2$	$[M+2Na-H]^+$
887.5692	887.5644	-5.4081	PI 38:4	$C_{47}H_{83}O_{13}P$	$[M+H]^+$
	887.562	-8.1121	PI 36:1	$C_{45}H_{85}O_{13}PNa$	$[M+Na]^+$
	887.5773	9.1260	PG 43:6	$C_{49}H_{85}O_{10}PNa$	$[M+Na]^+$
	887.5748	6.3093	PG 41:3	$C_{47}H_{87}O_{10}PNa_2$	$[M+2Na-H]^+$
901.5828	901.5801	-2.9947	PI 39:4	$C_{48}H_{85}O_{13}P$	$[M+H]^+$
	901.5776	-5.7677	PI 37:1	$C_{46}H_{87}O_{13}PNa$	$[M+Na]^+$
	901.5905	8.5404	PG 42:3	$C_{48}H_{89}O_{10}PNa_2$	$[M+2Na-H]^+$
915.5994	915.5957	-4.0411	PI 40:4	$C_{49}H_{87}O_{13}P$	$[M+H]^+$
	915.5933	-6.6623	PI 38:1	$C_{47}H_{89}O_{13}PNa$	$[M+Na]^+$
916.6041	916.6038	-0.3273	PS 44:5	$C_{50}H_{88}NO_{10}PNa$	$[M+Na]^+$
	916.6014	-2.9466	PS 42:2	$C_{48}H_{90}NO_{10}PNa_2$	$[M+2Na-H]^+$
929.6147	929.6114	-3.5499	PI 41:4	$C_{50}H_{89}O_{13}P$	$[M+H]^+$
	929.6089	-6.2392	PI 39:1	$C_{48}H_{91}O_{13}PNa$	$[M+Na]^+$
	929.6218	7.6375	PG 44:3	$C_{50}H_{93}O_{10}PNa_2$	$[M+2Na-H]^+$
931.5750	931.5672	-8.3730	PI 38:1	$C_{47}H_{89}O_{13}PK$	$[M+K]^+$
943.6275	943.627	-0.5298	PI 42:4	$C_{51}H_{91}O_{13}P$	$[M+H]^+$
	943.6246	-3.0733	PI 40:1	$C_{49}H_{93}O_{13}PNa$	$[M+Na]^+$

Supplementary Information Table 1 - Theoretical and observed masses in LAP-MALDI MS spectra for the intact doubly sodiated antibiotics, and the hydrolysed decarboxylated doubly sodiated antibiotic product, and respective ppm errors.

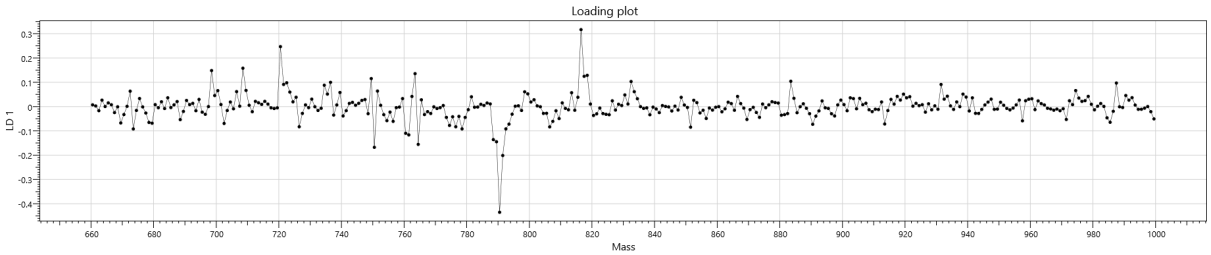
	Theoretical m/z value of $[M+2Na-H]^+$	Observed m/z value	Mass accuracy (ppm)
Ampicillin	394.0808	394.0770	-9.64
Ampicillin HD	368.1015	368.1003	-3.26
Cefalexin	392.0651	392.0634	-4.34
Cefalexin HD	366.0859	366.0862	0.82
Meropenem	428.1228	428.1214	-3.27
Meropenem HD	402.1434	402.1417	-4.23



Supplementary Information Figure 1 – Fragmentation of intact doubly sodiated meropenem and hydrolysed decarboxylated doubly sodiated meropenem via LAP-MALDI MS/MS.



Supplementary Figure 2 - LAP MALDI MS Spectra of susceptible (A) K. pneumoniae, susceptible K. pneumoniae spiked with penicillinase (B) and resistant K. pneumoniae, where ▲ indicates intact antibiotics, and ■ indicates hydrolysed decarboxylated antibiotic products.



Supplementary Information Figure 3 - Loading plot to show the peaks most responsible for variation in the first linear dimension of LDA, at m/z 795 and 818, in samples incubated with antibiotics.

Chapter 5 - LAP-MALDI MS profiling and identification of biomarkers for the detection of bovine tuberculosis

This work was part of an innovative tuberculosis diagnostics research project supported by the Department for Environment, Food & Rural Affairs through grant number 26952.

Research Article

Lellman SE, Reynolds CK, Jones AK, Taylor N and Cramer R. LAP-MALDI MS profiling and identification of biomarkers for the detection of bovine tuberculosis. Submitted to Journal of Agricultural and Food Chemistry on 24th March 2023. *Under revision.*

Author Contributions

Lellman SE – Design of experiment, undertaking investigation and analysis, drafting and editing the manuscript

Reynolds CK – Reviewing and editing the manuscript, expert subject matter advice, Co-Investigator

Jones AK – Sample collections and expert subject matter advice

Taylor N - Reviewing and editing the manuscript, expert subject matter advice

Cramer R – Conceptualisation, writing, reviewing, and editing the manuscript, Supervision, Principal Investigator

LAP-MALDI MS profiling and identification of biomarkers for the detection of bovine tuberculosis

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Abstract

Detecting bovine tuberculosis (bTB) primarily relies on the tuberculin skin test, requiring two separate animal handling events with a period of incubation time (normally 3 days) between them. Here, we present the use of liquid atmospheric pressure (LAP)-MALDI for the identification of bTB infection, employing a 3-class prediction model that was obtained by supervised linear discriminant analysis (LDA) and tested with bovine mastitis samples as disease-positive controls. Non-invasive collection of nasal swabs was used to collect samples, which were subsequently subjected to a short (<4 h) sample preparation method. Cross-validation of the 3-class LDA model from the processed nasal swabs provided a sensitivity of 75.0% and specificity of 91.4%, with an overall classification accuracy of 85.7%. These values are comparable to those for the skin test, showing that LAP-MALDI MS has the potential to provide an alternative single-visit diagnostic platform that can detect bTB within the same day of sampling.

1. Introduction

Bovine tuberculosis (bTB) is a world-wide disease that is devastating for the cattle population and has serious economic and social impacts for dairy farming, with significant risks to the human population through zoonotic transmission.(1) In Great Britain alone, 3,668 new herd incidents were reported between October 2021 and September 2022, with 76% of these being reported in the southwest and west of England, which is deemed a high-risk area by the UK's Government Department for Environment, Food and Rural Affairs (DEFRA).(2) In 2013, the UK Government launched various bTB eradication strategies, with the aim of declaring the UK bTB-free by 2038. The main priorities of this programme are the development of a cattle vaccine, enforcing wildlife control policies and improving diagnostic testing.

Overall, bTB costs the UK approximately £100 million per year, with over 27,000 cattle being slaughtered for disease control in 2021.(3) There are many factors that negatively influence the control of bTB. The causative bacterium, *Mycobacterium bovis*, has a complex life cycle. *M. bovis* can infect humans, as well as a wide range of animals, making it difficult to eradicate in British wildlife. In addition, infection with *M. bovis* is usually asymptomatic, with symptoms not presenting until late in disease progression, at the fatal stages of the disease.(3)

In the UK, there are currently two bTB diagnostic tests approved for use. The primary test is the single intradermal comparative cervical tuberculin (SICCT) test, also referred to as the tuberculin skin test, which measures a delayed hypersensitivity reaction in the animal.(4) Two individual injections of bovine and avian tuberculin are administered under the skin of the animal, and the test is read out 72 h later. If an inflammatory response to bovine

tuberculin relative to avian tuberculin is presented on the skin, this is deemed a positive test result. The secondary test is the interferon (IFN)-y blood test, where blood is drawn from the animal and mixed with bovine and avian tuberculin. The levels of cytokine produced in response are subsequently measured. The IFN-y test is used to supplement the tuberculin skin test, particularly in low-risk areas, to detect infections that may not have been detected simply with the skin test. The tuberculin skin test has a high specificity of 99.98%,(5) however the sensitivity is only approximately 80%.(6) It is for this reason that the IFN-y test supplements the tuberculin skin test, with a specificity of 96.6% and sensitivity of 87.6%.(7)

Alternative methods for bTB diagnostics have been investigated to improve the detection rate. Nucleic acid-based tests such as PCR testing have been used to target the *Mycobacterium tuberculosis* complex, which contains *M. bovis*, providing an average specificity of 97% and sensitivity of 87.7%.(8) However, the sample collection for this technique is invasive. Typically, the collection of tissue samples from lymph nodes is used, which is not suitable for large-scale diagnostics, and imposes additional distress to the animal. Point-of-care antigen tests, which were originally developed for humans, have also been tested against bTB. These have utilised various biological fluids to detect *M. tuberculosis*-specific antigens. However, further research is required on both as both tests have variable efficacies.(9)

MALDI mass spectrometry (MS) has been increasingly used for identification of bacterial infections in human and veterinary diagnostics. For both the same workflow is employed, whereby a clinical sample is obtained, and subsequent culturing is required for the growth/propagation of the pathogenic microorganisms.(10) However, there have been fewer advances in the rapid, direct analysis of clinical samples for veterinary diagnostics;

direct MALDI MS analysis of animal samples is more commonly applied to milk in the context of food adulteration.(11) The use of LAP-MALDI MS has recently been demonstrated for the detection of bovine mastitis with high specificity and sensitivity.(12) Only small volumes of milk are required for analysis, and using a quick preparation protocol, lipids, peptides and proteins can be detected within the mass spectral profile, allowing rapid diagnosis of mastitis two days before clinical manifestation.(13) LAP-MALDI MS contrasts to traditional MALDI,(14-16) in that liquid samples are analysed at atmospheric pressure, as opposed to solid, crystalline samples under vacuum, allowing simple sample preparation and introduction to the mass analyzer with less interference from matrix-cluster ions as is typically observed in traditional MALDI. It also allows the detection of ESI-like multiply charged ions in a low m/z range.(16, 17)

In this study, we present a novel application of LAP-MALDI MS profiling in veterinary diagnostics. It is shown that samples from cattle with bovine diseases such as bTB and bovine mastitis can be distinguished from samples of healthy cattle. Bovine samples were collected and prepared using a rapid (limited) digestion method, followed by analysis using LAP-MALDI MS. High specificity and sensitivity were obtained for the identification of bTB, mastitis and healthy bovine samples. This study was funded by the UK government as part of a 25-year initiative to eradicate bTB from the UK by 2038.

2. Materials and Methods

2.1 Materials

Cotton tipped wooden swabs, HPLC-grade water, ethanol, acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Loughborough, UK).

For the digestion, ammonium bicarbonate (ABC), dithiothreitol (DTT) and iodoacetamide (IAA) were bought from Sigma-Aldrich (Gillingham, UK). Sequencing-grade trypsin was purchased from Promega (Chilworth, UK) and C18 ZipTips for sample clean-up were purchased from Merck (Poole, UK).

For LAP-MALDI matrix preparation, α-cyano-4-hydroxycinnamic acid (CHCA) and propylene glycol were bought from Sigma-Aldrich.

2.2 Sample cohort

Sample collection for this study took place at seven different locations within the UK. Negative control samples were obtained from healthy animals. One of the sites used for the collection of negative controls was at Crichton Royal Farm in Dumfries, Scotland, which has been declared officially bTB-free since 2009. A second collection site for negative control samples was the Centre for Dairy Research (CEDAR) at the University of Reading (Reading, England), which was bTB-free at the time of sampling. The remaining negative controls were collected from farms in West Berkshire (England) on the readout day of tuberculin skin testing. These were sites where positive bTB skin tests were recorded on animals.

As (disease/infection-)positive controls for statistical modelling, swabs were also taken from cows diagnosed with mastitis, to determine whether differences are due to a general immune response, or are bTB-specific. All mastitis samples were collected from CEDAR.

The final class of samples was collected from bTB animals. Five of the bTB samples were obtained from reactor animals from two different farms in West Berkshire. These were collected at the readout stage of the tuberculin skin test. The remaining bTB samples were collected from naturally infected animals being held at the UK's Animal and Plant Health

Agency (APHA) at Weybridge (England). From these animals, a swab was taken from each nostril, totalling two swab samples per animal, except for one animal where only a single sample could be taken.

In total, 60 healthy samples were collected (negative controls), 22 bTB samples (positives) and 13 mastitis samples collected (disease-positive controls). Of these, 84 were from female cattle and 11 from male cattle. Details of all samples collected can be found in Supporting Information 1.

All reactors were confirmed via post-mortem or microbiological culture and were culled after sampling. With the exception of 14 healthy animals without any follow-up health information, all other animals sampled from, both healthy and those with mastitis, were otherwise healthy for at least 3 months following sampling (see Supporting Information 1).

2.3 Sample collection procedure

Nasal swabs were used for all sample collections. For each individual sample collection, a swab was inserted into one of the animal's nostrils for 3-5 seconds, ensuring the swab looked wet and was coated with nasal fluid. All swabs were triple-packaged and placed into an ice-filled freezer box for transportation to the laboratory. Upon receipt at the laboratory, samples were placed into a -80 °C freezer for storage. Supporting Information 2 provides details of the standard operating procedure (SOP) that was applied for the sample collection.

2.4 Sample preparation for MS analysis

Once all samples were collected, the samples were removed from the -80 °C freezer for batch processing. Swabs in their casing were immediately transferred into a microbiological

safety cabinet, removed from the outer casing, placed into a 1.5-mL tube containing 400 µL of 1X PBS, and briefly agitated at least 5 times to assist solubilisation of biomolecules. All swabs were gently squeezed against the inside walls of the 1.5-mL tube and subsequently discarded. A volume of 900 µL of 100% ethanol was added and the mixture was vortexed. As multiple samples were processed at the same time, samples were placed on ice at this stage. The sample mixtures were then centrifuged for 5 min at 13,000 rpm. The supernatant was removed and discarded, and the resultant pellet was resuspended in 30 µL of 0.1% TFA.

For the digestion, 50 µL of 50 mM ABC was added to the dissolved sample pellets and mixed by pipetting. For reduction, 5 µL of 100 mM DTT was added to the samples and vortexed, followed by incubation at 37 °C for 30 min. For subsequent alkylation, 10 µL of 100 mM IAA was added to the samples and vortexed, followed by incubation at room temperature in the dark for 30 min. For the next step of enzymatic digestion, a small volume of 2 µL containing 0.4 µg of trypsin was added and the samples were incubated at 37 °C for 2h. The digestion was stopped by acidification with 8 µL of 10% TFA. Samples were then purified using C18 ZipTips according to the manufacturer's instructions, with a final elution volume of 5 µL of ACN:0.1% TFA (1:1).

A liquid support matrix (LSM) was used for all LAP-MALDI MS measurements. It was formed of CHCA (25 mg/mL) in 70:30% (v/v) ACN:H_2O, subsequently adding PG in a ratio of 7:10 (PG:CHCA solution). A volume of 0.5 µL of LSM was spotted onto a stainless-steel MALDI sample plate, followed by the addition of 0.5 µL of the freshly prepared sample digest. Supporting Information 3 provides details for the SOP that was applied for the sample preparation and subsequent data acquisition and analysis.

2.5 MS and MS/MS data acquisition

All MS and MS/MS measurements were performed using a Synapt G2-Si (Waters; Wilmslow, UK) with an in-house built AP-MALDI source. Calibration of the instrument was performed using sodium iodide in the m/z region of 100-2000. The laser energy was set to 18 µJ/pulse, with a laser pulse repetition rate of 30 Hz. The ion source was operated in positive ion mode, at 3.0 kV with a counter nitrogen gas flow of 180 L/h, and heated capillary. All data acquisition and initial data processing were performed using MassLynx 4.2® software (Waters). Data acquisition for each sample was for 1 minute with 1 scan per second.

MS/MS data acquisition was performed in mobility TOF mode. Precursor ions were selected and the quadrupole isolation window was adjusted using LM and HM resolution values, dependent on the precursor ions. Multiple charge states were sequentially selected for fragmentation using collision-induced dissociation (CID). The collision voltage was set at 40 V in the trap cell for the 10+ charge state. Further CID fragmentation spectra were acquired in the transfer cell. The collision voltage varied before 30 V and 60 V, depending on the charge state selected for fragmentation.

2.6 MS Data Analysis

Statistical analysis of the MS profiles was performed with Abstract Model Builder (AMX; [Beta] Version 1.0.1962.0; Waters). All data files were imported to the AMX software, and spectra from all scans per file were selected. For all data files, binning of mass spectral data was performed every unit value in the m/z range of 700-1800. Linear discriminant analysis (LDA) was selected for all analyses with a pre-processing method using principal component analysis (PCA) for dimensionality reduction. Following PCA, LDA was applied to determine the maximum variation between the applied classes of sample (‘Healthy’, ‘bTB’ and ‘Mastitis’ or simply ‘Healthy’ and ‘Diseased’). Cross-validation of the LDA models was

performed using the built-in '20% out' function, with outliers based upon five standard deviations. For PCA, 50 dimensions were chosen, while for LDA the number of dimensions was 1 and 2 for the 2-class and 3-class analysis, respectively. Outliers were defined by 5 standard deviations.

2.7 MS/MS Data Analysis

Ion mobility filtering was applied post-acquisition in order to remove interfering singly charged ions from the mass spectrum. A band selection was applied, and the data was exported to MassLynx, retaining the drift time.

As all data was acquired in mobility TOF mode, the fragment ion peak list was created manually (due to file compatibility reasons) and searched using MS/MS Ion Search of the MASCOT search software (version 2.7; Matrix Science; London, UK). For the identification of larger proteins, only the singly charged fragment ions that were common to more than one fragmentation spectrum were included in the peak list. All multiply charged fragment ions of each fragmentation spectrum obtained from the different multiple charge states were also included in the peak list as $[M+H]^+$ ions. To obtain the mass values for the $[M+H]^+$ ions, the multiply charged fragment ion signals were deconvoluted using the MaxEnt plug-in for MassLynx, with deconvoluted molecular mass range of 100-11,000 Da and a maximum of 10 charges. Deconvoluted signals of the fragment ions obtained by MaxEnt were verified by checking the actual MS/MS spectra for their appearances. Fragment ion peak lists were searched against the NCBIprot database (version 20201010) with ±75 ppm peptide mass tolerance and ±0.2 Da fragment mass tolerance. Larger proteins were searched with 'NoCleave' as the enzyme. Presumed tryptic peptides were searched using trypsin as the

enzyme, allowing for 1 missed cleavage, with carbamidomethyl as a fixed modification and oxidation (M) and acetyl (N-term) selected as variable modifications.

3. Results

Due to varying collection dates, all swabs were stored at -80 °C in quarantine until all swabs had been collected, in order to process all samples at the same time. When all samples were collected, the swabs underwent a simple precipitation procedure using ethanol to concentrate biomolecules within the sample as well as for the inactivation of *M. bovis* and any other hazardous microorganisms that may be present, for health and safety purposes. Samples were spun down and the pellets resuspended in 0.1% TFA. In preliminary testing, the analysis of the resolubilised pellets did not yield informative results, and therefore a short enzymatic digestion step was added. The use of LAP-MALDI MS is somewhat limited in the detection of larger biomolecules. Hence, a digestion step was added in order to cleave any larger proteins into smaller fragments, facilitating their detection by LAP-MALDI MS.

All LAP-MALDI samples were spotted onto a 96-well MALDI sample plate and analysed sequentially by acquiring MS profiles in the m/z range of 100-2000. Following the acquisition of the MS profile data, the data files were imported into AMX model builder for statistical analysis. The m/z region below 700 was not included in the data analysis for class modelling as there are only a few analytes of interest in this region, while ion signals from the MALDI matrix and contaminations can be present to a greater extent, thus limiting the influence of non-specific ion signals on the data modelling.

Both LDA and PCA were applied to the obtained MS profile data set. LDA is a supervised technique, taking into account the assigned classes prior to building a classification model, maximising the difference between the classes. PCA is an unsupervised technique, maximising variation in the whole data set without using any known class labels. PCA was used in this study to evaluate whether any principal component could be found that can easily cluster the profile data according to other variables than the health status, in particular the geographic location of the animals. For this, only the profiles of healthy animals were interrogated. Figure 1 shows a visualisation of the obtained PCA data, whereby through cross-validation all samples were classified as outliers.

Using the entire profile data set (limited to the m/z region of 700-1800), LDA was used for building prediction models to classify healthy (negative controls), mastitis (positive controls) and bTB animals. Figure 2 presents the obtained LDA classification data, demonstrating an overall classification accuracy of 85.7% with a bTB detection sensitivity of 75.0% and specificity of 90.1% when applying the '20% out' cross-validation (excluding four outliers, whose analysis would be repeated in routine testing). PCA was also performed on this data set, leading to no outliers but providing a lower cross-validation accuracy of 72% compared to LDA.

The class labels were then simplified to healthy (negative control) and diseased (mastitis or bTB). For this 2-class system, the cross-validated LDA model provided an overall classification accuracy of 88.4% with a sensitivity of 88.6% and specificity of 88.3% (Figure 3).

From the loading plot (Figure 4), one putative biomarker protein was identified as being highly responsible for the variation in the dataset, belonging to the diseased animals. This

protein's assigned ions were fragmented by CID and analysed using top-down LAP-MALDI MS/MS as described in section 2.7. The fragment ion peak list used for database searching can be found in Supporting Information 4 and the fragmentation spectrum of the $[M+10H]^{10+}$ ions can be found in Supporting Information 5. The obtained MS/MS data was searched against the NCBIprot protein database as described in section 2.7, allowing for all taxonomies. The only significant hits obtained from this search were for bovine proteoforms of S100-A12 (NCBIprot accession number NP_777076), which is a protein that is released by inflammatory cells in response to environmental cues.

4. Discussion

Current bTB testing typically employs methods that are invasive such as the tuberculin skin test and the IFN-y blood test. Both tests are invasive and take their time, typically 72h for the tuberculin skin test, requiring two farm visits, reagents and consumables that add to the overall costs of the test. Thus, less invasive and faster tests while being more cost-effective would improve bTB detection and disease management.

Earlier studies analysing non-invasively collected milk from dairy cows showed that a simple one-pot sample preparation without any disease-specific reagents is all that is needed for preparing samples to detect mastitis by LAP-MALDI MS profiling.(12) Further method development and application to a larger, longitudinally collected sample set of bovine milk demonstrated that LAP-MALDI MS profiling was able to detect mastitis up to 2 days before its clinical detection. The cost for large-scale application based on daily sampling of large herds was calculated to be less than US$0.1 per sample.(13)

In this present study, a similar analytical approach was employed by utilizing LAP-MALDI MS profiling. However, no blood or milk but the nasal fluids from the cattle's nostrils were collected, being less invasive than current bTB tests and allowing disease detection for male animals. Compared to the analysis of milk by LAP-MALDI MS, a wooden swab was used to collect the sample and a short, limited proteolysis step was added to the sample preparation. The latter required no disease-specific reagents and added only marginally to the consumables costs when compared to current bTB tests. After the collected samples reach the analytical laboratory, the total sample preparation and analysis time (to result) can be as short as 4 h, substantially faster than the tuberculin skin test. The LAP-MALDI MS platform is also capable of high throughput analysis,(18, 19) and large-scale population screening is therefore a possibility.

Although the data obtained so far is still limited by the number of bTB animals and overall sample numbers, it clearly demonstrates the potential of LAP-MALDI MS for disease classification beyond the detection of mastitis from fluids that are not as rich as milk with respect to disease-specific biomarkers. For the 3-class model, the sample set provided an overall classification accuracy of 85.7%, with a bTB detection sensitivity and specificity of 75.0% and 90.1%, respectively. For simply classifying healthy vs diseased, the accuracy was 88.4%, with a sensitivity of 88.6% and specificity of 88.3%. A review of bTB testing methods showed that most of the reviewed tuberculin skin test studies had an extremely high specificity of typically 90-100% but a much lower sensitivity, being therefore described as "imperfect". (20) The sensitivity and specificity values obtained with LAP-MALDI MS profiling are similar but with the potential to further improve once the prediction model has been refined by a larger data set.

In this context, it should be noted that the outliers obtained by the 3-class LDA model are based upon 5 standard deviations and were excluded from the final percentage values that are presented within this article. In 'real-life' testing, samples classed as outliers would be re-analysed. Only in the case that a sample is still classified as an outlier, would the animal have to be swabbed again.

The loading plot for the 2-class LDA analysis (Figure 4) shows that one protein has a large influence on the variation in principal component 1, which accounts for 22.39% of the variation within the 2-class model. Loading plots are typically used in unsupervised statistical analysis in order to reveal the peaks most responsible for the variation in the data set. With the AMX software, the data is initially linearised into principal components, followed by the application of the class labels and subsequent LDA. A loading plot can then be viewed for the underlying principal components used in the LDA. From this loading plot and subsequent LAP-MALDI MS/MS analysis, protein S100-A12 was identified as a key protein responsible for the discrimination between healthy and diseased states.

Despite the applied (though limited) digestion step S100-A12 was identified in the MS profile as the full-length protein with the N-terminal (initiator) methionine removed. After the digestion step, there were many doubly charged peptides in the LAP-MALDI mass spectrum which suggests that the digestion was successful for other proteins that were in the sample. Some of these were identified as tryptic peptides from various sources, including bovine IgA (NCBIprot accession number G3MXB5) and rape seed storage protein (NCBIprot accession number CDY29281.1). That S100-A12 was detected as virtually intact protein despite the digestion step can be explained by its known resistance to protease digestion,(21) with many of the lysine residues being located next to aspartic acid residues.

Trypsin digestions can take longer when lysine and arginine residues are located next to acidic amino acids.(22) Interestingly, it was not possible to detect the protein without the limited digestion step.

S100-A12 is known to bind to a receptor for advanced glycation end-products (RAGE), whose activation leads to pro-inflammatory effects.(21) In humans, S100-A12 is implicated in many diseases, including coronary heart disease,(23) periodontitis,(24) as well as lung disease, including pulmonary tuberculosis.(25) S100-A12 has been previously detected in bovine milk, and described as a marker of sub-clinical mastitis,(26) which is supported by the data obtained here. It has also been reported in cows infected with *Mycobacterium avium* ssp. *paratuberculosis.*(27) The importance of S100-A12 in response to many diseases, including infectious diseases such as bovine mastitis, and infections with *M. avium* is in good agreement with its identification as a disease marker in this study.

The aim of this study was to evaluate and adopt the LAP-MALDI MS platform for its application to bTB detection. In comparison to current first-line testing using the tuberculin skin test, the results of this study showed a similar sensitivity and specificity with a much faster procedure and a less invasive farm-site sample collection. The acquisition of nasal swabs can be performed by trained veterinary or farm staff whilst cattle are safely restrained within a cattle crush (squeeze chute). The nasal fluid collection is fast, and the required sample volumes are low (<0.5mL).

Given the proof-of-principle nature of this study and the limited sample set, further improvements can be expected. To develop this method further, a wider and larger sample set is desirable, with a greater variety of breeds, geographical locations and other diseases. In particular, diseases that are closely related to bTB such as Johne's disease or other

respiratory diseases. However, LAP-MALDI MS profiling and subsequent LAP-MALDI MS/MS analyses from the same samples used for profiling have demonstrated that this approach is based on the detection of disease-relevant biomarkers such as S100-A12. These and others from further combined LAP-MALDI MS profiling and MS/MS analyses could ultimately provide individual biomarkers or panels of biomarkers that are highly disease-specific, which could also be exploited for lateral flow antigen tests.

The ability to distinguish between healthy cattle, as well as two disease states, shows the potential for this platform to be used in multiplex analyses, making it highly versatile and even more cost-effective. Due to its simplicity, speed and low consumable costs there is also the potential for large-scale population screening. Similar to the tuberculin skin test, LAP-MALDI MS profiling could be employed in first-line testing, followed by further testing modalities as it is currently the case with bTB testing. Its speed, i.e. faster read-out, might also make it an attractive proposition for earlier intervention and disease management, even if the test accuracy cannot be further improved or will be ultimately lower than for the tuberculin skin test.

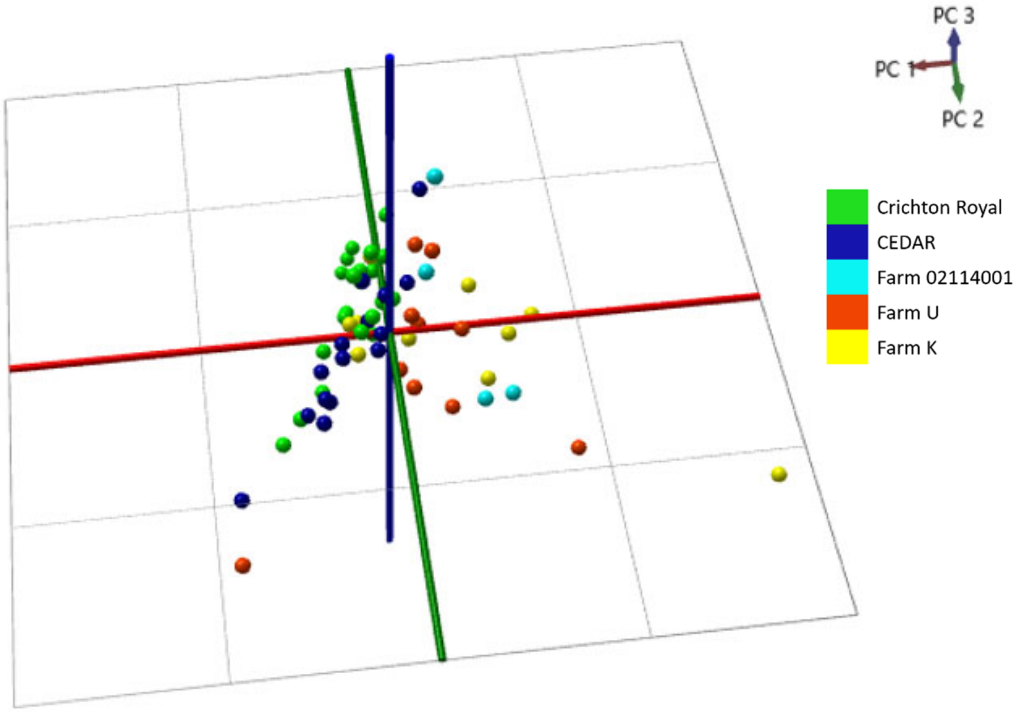
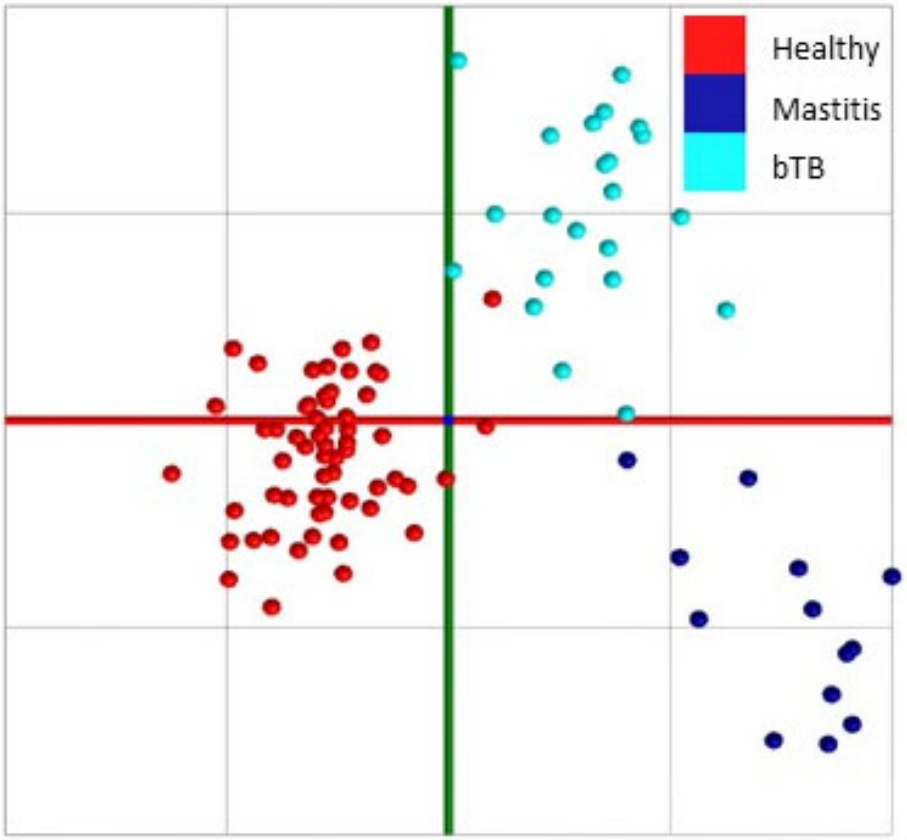


Figure 1 – Visualisation of PCA of samples from healthy animals to determine any bias due to their geographical location. Each geographical location is represented by a different colour.

A

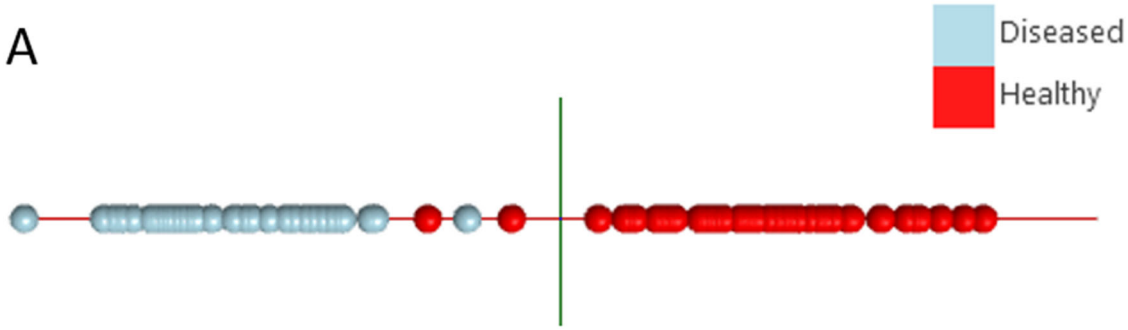


B

	Assigned Healthy	Assigned Mastitis	Assigned bTB	Outlier	Total
Confirmed Healthy	53	0	6	1	60
Confirmed Mastitis	1	10	1	1	13
Confirmed bTB	4	1	15	2	22
Total	58	11	22	4	95

Figure 2 – (A) Visualisation of the linear discriminant analysis (LDA) for discrimination between healthy, mastitis and bTB samples. (B) Confusion matrix detailing the assignments based upon the LDA model in (A), using '20% out' cross-validation.

A



B

	Assigned Diseased	Assigned Healthy	Total
Confirmed Diseased	31	4	35
Confirmed Healthy	7	53	60
Total	38	57	95

Figure 3 – (A) Visualisation of the linear discriminant analysis (LDA) for the discrimination between samples from healthy and samples from diseased (bTB and mastitis) animals. (B) Confusion matrix detailing the identification of samples from healthy and diseased animals based upon the LDA model in (A), using '20% out' cross validation.

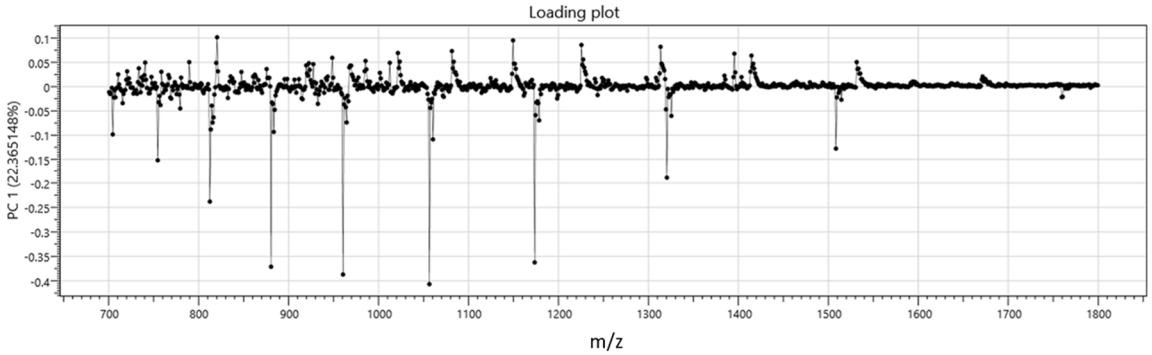


Figure 4 – Loading plot for the first principal component in the 2-class LDA model, indicating the peaks most responsible for discrimination.

Supporting Information

S1 – List of animals and their follow-up health status; S2 – SOP for Nasal Swab Sample Collection; S3 – SOP for Nasal Swab Analysis by LAP MALDI MS; S4 – Peak List used for Identifying S100-A12; S5 - Fragmentation Spectrum of the $[M+10H]^{10+}$ Ion of S100-A12 (PDF)

Data Availability

Data supporting the results reported in this paper are openly available from the University of Reading Research Data Archive at https://doi.org/10.17864/1947.000443.

Acknowledgements

We kindly thank Waters Corporation for access to the AMX [Beta] software. We also thank APHA, Crichton Royal Farm and the local farms for collecting and donating samples for this study, as well as DEFRA who provided funding (ref. 26952), continuous support throughout the project and assistance in obtaining bTB animal samples. This work was supported by EPSRC through grant EP/R513301/1.

References

1. Müller B, Dürr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerging Infectious Diseases. 2013;19(6):899-908.
2. Department for Environment Food & Rural Affairs. Historical statistics notices on the incidence of tuberculosis (TB) in cattle in Great Britain, 2022 - quarterly: https://www.gov.uk/government/statistics/historical-statistics-notices-on-the-incidence-of-tuberculosis-tb-in-cattle-in-great-britain-2022-quarterly/figures-to-september-2022-published-14-december-2022; 2022 [Accessed 05 March 2023].

3. Brooks-Pollock E, Roberts GO, Keeling MJ. A dynamic model of bovine tuberculosis spread and control in Great Britain. Nature. 2014;511(7508):228-31.
4. Monaghan ML, Doherty ML, Collins JD, Kazda JF, Quinn PJ. The tuberculin test. Veterinary Microbiology. 1994;40(1):111-24.
5. Goodchild AV, Downs SH, Upton P, Wood JL, de la Rua-Domenech R. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Veterinary Record. 2015;177(10):258.
6. Praud A, Boschiroli ML, Meyer L, Garin-Bastuji B, Dufour B. Assessment of the sensitivity of the gamma-interferon test and the single intradermal comparative cervical test for the diagnosis of bovine tuberculosis under field conditions. Epidemiology and Infection. 2015;143(1):157-66.
7. Gormley E, Doyle M, Duignan A, Good M, More SJ, Clegg TA. Identification of risk factors associated with disclosure of false positive bovine tuberculosis reactors using the gamma-interferon (IFNγ) assay. Veterinary Research. 2013;44(1):117.
8. Courcoul A, Moyen J-L, Brugère L, Faye S, Hénault S, Gares H, et al. Estimation of Sensitivity and Specificity of Bacteriology, Histopathology and PCR for the Confirmatory Diagnosis of Bovine Tuberculosis Using Latent Class Analysis. PLOS ONE. 2014;9(3):e90334.
9. Kelley HV, Waibel SM, Sidiki S, Tomatis-Souverbielle C, Scordo JM, Hunt WG, et al. Accuracy of Two Point-of-Care Tests for Rapid Diagnosis of Bovine Tuberculosis at Animal Level using Non-Invasive Specimens. Scientific Reports. 2020;10(1):5441.
10. Randall LP, Lemma F, Koylass M, Rogers J, Ayling RD, Worth D, et al. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. Research in Veterinary Science. 2015;101:42-9.
11. Sassi M, Arena S, Scaloni A. MALDI-TOF-MS Platform for Integrated Proteomic and Peptidomic Profiling of Milk Samples Allows Rapid Detection of Food Adulterations. Journal of Agricultural and Food Chemistry. 2015;63(27):6157-71.
12. Hale OJ, Morris M, Jones B, Reynolds CK, Cramer R. Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Adds Enhanced Functionalities to MALDI MS Profiling for Disease Diagnostics. ACS Omega. 2019;4(7):12759-65.
13. Piras C, Hale OJ, Reynolds CK, Jones AK, Taylor N, Morris M, et al. LAP-MALDI MS coupled with machine learning: an ambient mass spectrometry approach for high-throughput diagnostics. Chemical Science. 2022;13(6):1746-58.
14. Ryumin P, Brown J, Morris M, Cramer R. Protein identification using a nanoUHPLC-AP-MALDI MS/MS workflow with CID of multiply charged proteolytic peptides. International Journal of Mass Spectrometry. 2017;416:20-8.
15. Ryumin P, Cramer R. The composition of liquid atmospheric pressure matrix-assisted laser desorption/ionization matrices and its effect on ionization in mass spectrometry. Analytica Chimica Acta. 2018;1013:43-53.
16. Cramer R, Pirkl A, Hillenkamp F, Dreisewerd K. Liquid AP-UV-MALDI enables stable ion yields of multiply charged peptide and protein ions for sensitive analysis by mass spectrometry. Angewandte Chemie International Edition English. 2013;52(8):2364-7.
17. Ryumin P, Brown J, Morris M, Cramer R. Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS. Methods. 2016;104:11-20.
18. Krenkel H, Hartmane E, Piras C, Brown J, Morris M, Cramer R. Advancing Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Toward Ultrahigh-Throughput Analysis. Analytical Chemistry. 2020;92(4):2931-6.
19. Krenkel H, Brown J, Richardson K, Hoyes E, Morris M, Cramer R. Ultrahigh-Throughput Sample Analysis Using Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Analytical Chemistry. 2022;94(10):4141-5.
20. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Research in Veterinary Science. 2006;81(2):190-210.

21. Nacken W, Kerkhoff C. The hetero-oligomeric complex of the S100A8/S100A9 protein is extremely protease resistant. FEBS Letters. 2007;581(26):5127-30.
22. Simpson RJ. Fragmentation of protein using trypsin. CSH Protocols. 2006;2006(5).
23. Goyette J, Yan WX, Yamen E, Chung YM, Lim SY, Hsu K, et al. Pleiotropic Roles of S100A12 in Coronary Atherosclerotic Plaque Formation and Rupture1. The Journal of Immunology. 2009;183(1):593-603.
24. Lira-Junior R, Holmström SB, Clark R, Zwicker S, Majster M, Johannsen G, et al. S100A12 Expression Is Modulated During Monocyte Differentiation and Reflects Periodontitis Severity. Frontiers in Immunology. 2020;11.
25. Smirnov IP, Zhu X, Taylor T, Huang Y, Ross P, Papayanopoulos IA, et al. Suppression of alpha-cyano-4-hydroxycinnamic acid matrix clusters and reduction of chemical noise in MALDI-TOF mass spectrometry. Analytical Chemistry. 2004;76(10):2958-65.
26. Zhong K, Zhang CY, Zha GM, Wang XJ, Jiao XQ, Zhu HS, et al. S100 calcium-binding protein A12 as a diagnostic index for subclinical mastitis in cows. Reproduction in Domestic Animals. 2018;53(6):1442-7.
27. Verschoor CP, Pant SD, You Q, Kelton DF, Karrow NA. Gene expression profiling of PBMCs from Holstein and Jersey cows sub-clinically infected with Mycobacterium avium ssp. paratuberculosis. Veterinary Immunology and Immunopathology. 2010;137(1):1-11.

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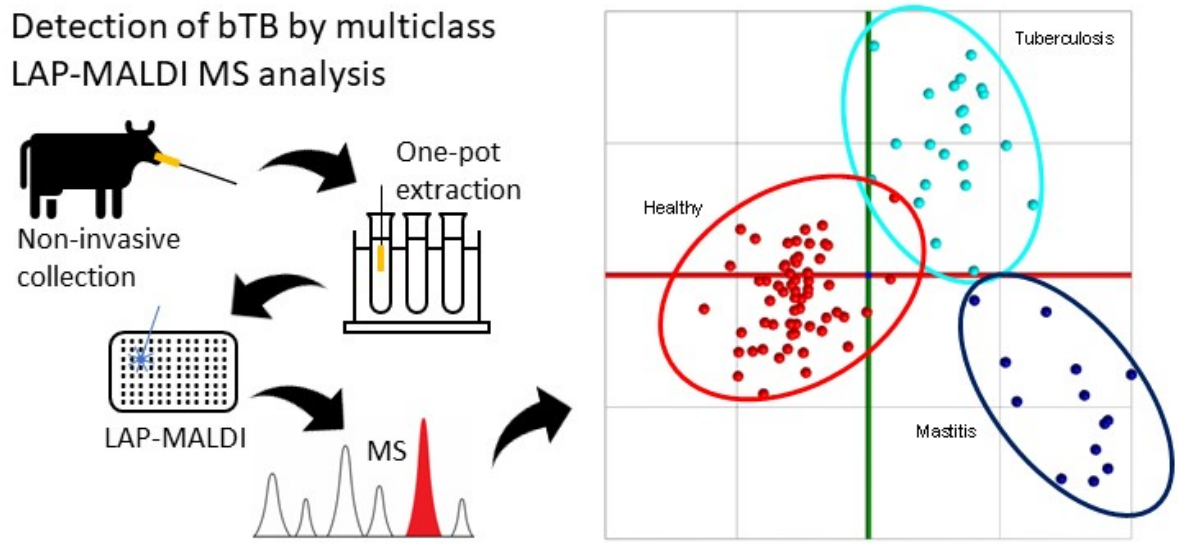

Supporting Information

LAP-MALDI MS profiling and identification of biomarkers for the detection of bovine tuberculosis

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Contents

Supporting Information 1 – List of Animals and their Follow-up Health Status

Source	Animal ID	Sample Type	Sample Code	Sex	Breed	Follow-Up Health Status
APHA	C21-2488L	Reactor	APHAC21-2488L	Female	Holstein	Culled
APHA	C21-2488R	Reactor	APHAC21-2488R	Female	Holstein	Culled
APHA	C21-2485L	Reactor	APHAC21-2485L	Female	Holstein	Culled
APHA	C21-2485R	Reactor	APHAC21-2485R	Female	Holstein	Culled
APHA	C21-2487L	Reactor	APHAC21-2487L	Female	Holstein	Culled
APHA	C21-2487R	Reactor	APHAC21-2487R	Female	Holstein	Culled
APHA	C21-2486L	Reactor	APHAC21-2486L	Female	Holstein	Culled
APHA	C21-2486R	Reactor	APHAC21-2486R	Female	Holstein	Culled
APHA	2559L	Reactor	APHA2559L	Female	Holstein	Culled
APHA	2560L	Reactor	APHA2560L	Female	Holstein	Culled
APHA	2560R	Reactor	APHA2560R	Female	Holstein	Culled
APHA	2561L	Reactor	APHA2561L	Male	Holstein	Culled
APHA	2561R	Reactor	APHA2561R	Male	Holstein	Culled
APHA	2562L	Reactor	APHA2562L	Female	Holstein	Culled
APHA	2562R	Reactor	APHA2562R	Female	Holstein	Culled
APHA	2563L	Reactor	APHA2563L	Female	Holstein	Culled
APHA	2563R	Reactor	APHA2563R	Female	Holstein	Culled
Farm 021140001	1	Control	02114001C1	Female	Holstein	Unknown
Farm 021140001	2	Control	02114991C2	Female	Holstein	Unknown
Farm 021140001	3	Control	02114991C3	Female	Holstein	Unknown
Farm 021140001	4	Control	02114991C4	Female	Holstein	Unknown
Farm 021140001	50	Reactor	02114991R050	Female	Holstein	Culled
Farm 021140001	343	Reactor	02114991R343	Female	Holstein	Culled
Farm 021140001	3240	Reactor	02114991R3240	Female	Holstein	Culled
Farm 021140001	2637	Reactor	02114991R2637	Female	Holstein	Culled
Farm F	307406	Reactor	F-R307406	Female	Holstein	Culled
Farm K	400614	Control	KFC400614	Female	Charolais	Healthy
Farm K	300963	Control	KFC300963	Female	Charolais	Healthy
Farm K	200969	Control	KFC200969	Female	Charolais	Healthy
Farm K	400901	Control	KFC400901	Female	Charolais	Healthy
Farm K	600973	Control	KFC600973	Female	Charolais	Healthy
Farm K	700981	Control	KFC700981	Female	Charolais	Healthy
Farm K	300956	Control	KFC300956	Female	Charolais	Healthy
Farm K	300935	Control	KFC300935	Female	Charolais	Healthy
Farm K	00090	Control	KFC00090	Female	Charolais	Healthy
Farm K	600504	Control	KFC600504	Female	Charolais	Healthy

Farm U	01	Control	UKNC01	Female		Unknown
Farm U	02	Control	UKNC02	Female		Unknown
Farm U	03	Control	UKNC03	Female		Unknown
Farm U	04	Control	UKNC04	Female		Unknown
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Farm U	06	Control	UKNC06	Female		Unknown
Farm U	07	Control	UKNC07	Female		Unknown
Farm U	08	Control	UKNC08	Female		Unknown
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Farm U	10	Control	UKNC10	Female		Unknown
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CEDAR	7047	Control	CEDAR7047	Male	Angus	Healthy
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CEDAR	7050	Control	CEDAR7050	Male	Angus	Healthy
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Crichton Royal	200	Control	CRC200	Female	Holstein Freesian	Healthy

Supporting Information 2 – SOP for Nasal Swab Sample Collection

Standard Operating Procedure (SOP) for oral and nasal swab collection for the diagnosis of bovine tuberculosis (bTB)

Version 1.3 (24th November 2021)

Barney Jones, Sophie Lellman and Rainer Cramer
Department of Chemistry, UoR

Contents

1. Purpose

The purpose of this document is to provide guidance for sample collection of oral and nasal swabs from cattle for the further downstream processing and analysis by mass spectrometry.

2. Scope

This SOP is designed for the appropriate collection of nasal and oral swabs from cattle for disease diagnostics. Sample collection is performed by trained research, farm or veterinary staff, in many cases at the time of reading the tuberculin skin test. In order to efficiently detect biomarkers for indicators of disease it is essential that a robust sample preparation protocol is established. In some cases, these biomarkers may be present in very small quantities. The presence of an established SOP will minimise the effects of user variability during sample collection.

Disclaimer: *This protocol has been developed and tested for research use only, not for veterinary or clinical use. No claims are made for its usefulness, accuracy or safety, and no liability can be accepted for any damages, losses or other expenses of any nature whatsoever arising from its use or supply. This protocol does not cover any legal or ethical issues.*

3. Health and Safety

Handle all swab samples as a potential source of pathogens, use appropriate personal protective equipment and handle samples as potential carriers of (bio)hazardous materials. Users of this protocol should have read and understood all relevant Risk and COSHH Assessments and other relevant SOPs, in particular health and safety rules and regulations and SOPs for handling farm animals and their body fluids. Before attempting to use this SOP, it is important to read and understand this document in its entirety.

4. Recommended equipment/material and personnel required (per collection event)

4.1 Equipment

- Polystyrene transport box (35cm*35cm*35cm) filled with ice
- Black permanent marker
- Grip seal plastic bag
- Storage freezer box

4.2 Disposables

- Plain wood stick swabs with tube casing (Thermo Scientific™, F150CA SWA2999)

4.3 Personnel required

- Two trained research, farm or veterinary staff will be required for the whole duration of each sampling. This is necessary to ensure the safety of personnel and the safety and welfare of the animals being sampled.

5. Procedural guidelines

Only collect oral and nasal swabs whilst the animal is physically restrained in a cattle crush. The animal should then be haltered and tied to the side of the crush in such a way that the head cannot move. This is essential both for the safety of the staff taking the swabs and for the safety of the animal as there is a danger the swab could break off in nasal cavity or mouth/pharynx. Only experienced staff members should attempt to restrain a cow as inexperience leads to dangers for all involved.
Once the animal is secure within the crush and correctly haltered, the procedure of taking the swab can be followed.

5.1 Nasal Swab Sample Collection Procedure

5.1.1 Clean nostrils provide the best nasal samples. However, traces of dirt or feed in the animal's nostrils is acceptable though not desirable. Wherever possible, avoid collecting swab samples immediately after the animal has eaten. Ensure the collection end of the swab does not touch anything other than the animal's nostril to prevent contamination of the sample.

5.1.2 Holding the tube, unscrew the lid and carefully remove the swab. Immediately, rub the end containing the swab inside the animal's nostril for 3-5 seconds. Ensure the swab looks wet and is coated with the nasal sample to achieve best results.

5.1.3 Holding the tube in one hand, carefully insert the nasal swab into the tube. Screw cap on tightly to prevent any leakage during transport.

5.1.4 Using a permanent marker, clearly write the animal identification number, farm identification code and the type of swab (nasal or oral) on the side of the tube and place the tube in a grip seal bag and immediately on ice.

5.1.5 Go to 5.2 for oral swab sample collection.

5.2 Oral Swab Sample Collection Procedure

5.2.1 As before, avoid collecting swab samples immediately after the animal has eaten. Try to avoid traces of dirt or feed. Ensure the collection end of the swab does not touch anything other than the animal's mouth to prevent contamination of the sample.

5.2.2 Holding the tube, unscrew the lid and carefully remove the swab. Immediately, rub the end containing the swab on the inside base of the animal's mouth to coat in saliva for 5 seconds. Please note that too much pressure can result in swab breaking. Ensure the swab looks wet and is coated with the saliva sample to achieve best results.

5.2.3 Holding the tube in one hand, carefully insert the swab into the tube. Screw cap on tightly to prevent any leakage during transport.

5.2.4 Using a permanent marker, clearly write the animal identification number, farm identification code and the type of swab (nasal or oral) on the side of the tube and place the tube in a grip seal bag and immediately on ice.

5.3 Packaging for Transport

5.3.1 Following swab sample collection, the grip seal bags containing the swabs are placed into an ice-filled freezer transport box with the lid securely on for transportation.

5.4 Receipt of Samples in the Laboratory

5.4.1 Trained and vaccinated personnel will receive samples delivered to the Chemistry building and transport them straight to laboratory room 142 in the Chemistry building.

5.4.2 For samples to be stored, swabs will be stored in sample quarantine within a storage freezer box in a locked -80°C freezer in laboratory room 142 until required for sample processing.

5.4.3 For further processing, samples are moved into a microbiological safety cabinet where the packaging can be removed, making the sample accessible for downstream processing. Follow all relevant risk assessment for handling and further processing of the samples.

Supporting Information 3 – SOP for Nasal Swab Analysis by LAP MALDI MS

Standard Operating Procedure (SOP) for nasal swab processing for the detection of disease in cattle by LAP-MALDI mass spectrometry analysis

Version 1.0 (March 2022)

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1. Purpose

The purpose of this document is to provide guidance for the processing of nasal swabs from cattle for analysis by liquid atmospheric pressure (LAP)-MALDI mass spectrometry (MS), and the subsequent data analysis and mining, for the detection of bovine tuberculosis (bTB).

2. Scope

This SOP has been designed for the appropriate processing of nasal swabs from cattle for the detection of bTB and potentially other diseases. Nasal swabs have been previously collected by trained research, farm or veterinary staff (see *Standard Operating Procedure (SOP) for oral and nasal swab collection for the diagnosis of bovine tuberculosis (bTB)* – version 1.3). The sample preparation protocol has been optimised to include a short digestion step to assist in the detection of biomarkers for disease classification. The following protocol is designed for data acquisition using LAP-MALDI MS and subsequent data analysis.

Disclaimer: *This protocol has been developed and tested for research use only, not for veterinary or clinical use. No claims are made for its usefulness, accuracy or safety, and no liability can be accepted for any damages, losses or other expenses of any nature whatsoever arising from its use or supply. This protocol does not cover any legal or ethical issues.*

3. Health and Safety

Swab samples to be used following this SOP will contain pathogens including causative agents of bTB (*Mycobacterium bovis*) and bovine mastitis (including but not limited to *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* and *Streptococcus uberis*). *M. bovis* is a biosafety level 3 bacterium and therefore must be handled in a category 3 laboratory. A derogation from category 3 to category 2 for the handling of *M. bovis* containing samples can be applied for from HSE providing suitable health and safety measures. Bacterial species causing bovine mastitis are biosafety level 2 pathogens and can therefore be handled in a category 2 facility.

All swab samples, whether health status is known or not, should be handled as a potential source of pathogens, using appropriate personal protective equipment, and handled as potential carriers of (bio)hazardous materials. Users of this protocol should have read and understood all relevant Risk and COSHH Assessments and other relevant SOPs, in particular health and safety rules and regulations and SOPs for handling farm animals and their body fluids. Before attempting to use this SOP, it is important to read and understand this document in its entirety and ensure all suitable H&S procedures are in place before commencing.

4. Recommended equipment/material and personnel required

a. Equipment

- Bench-top centrifuge capable of 13,000 rpm with rotor for 1.5mL tubes
- Incubator oven set at 37°C
- Standard lab vortexer

- Synapt G2-Si mass spectrometer (Waters Corporation, Wilmslow, UK) with AP-MALDI source (detailed in Ryumin et al., 2016) and MALDI sample plate
- Microbiological safety cabinet (see section 3 for further details)

b. Disposables

- 1.5-mL tubes (Fisher Scientific, 05-408-129)
- C18 ZipTips (Merck, ZTC18S096)
- 0.1-10-µL pipette tips (Fisher Scientific, 0030073428)
- 2-200-µL pipette tips (Fisher Scientific, 00300734363)
- 5-1000-µL pipette tips (Fisher Scientific, 0030073460)

c. Chemicals and Reagents

- 1x Phosphate-Buffered Saline (PBS; prepared according to the 1X PBS recipe: https://www.sigmaaldrich.com/GB/en/support/calculators-and-apps/1x-phosphate-buffered-saline)
- Ethanol (Sigma-Aldrich, 32221-2.5L-M)
- Water, HPLC Grade (Fisher Scientific, W/0106/17)
- Acetonitrile (ACN), HPLC Grade (Honeywell, 608-001-00-3)
- Trifluoroacetic acid (TFA), LC-MS Grade (TFA) (Thermo Scientific, 85183)
- Ammonium bicarbonate (Sigma-Aldrich, 09830-500G) buffer, 50mM in HPLC-Grade H_2O
- Dithiothreitol (DTT) (Sigma-Aldrich, D0632-1G)
- Iodoacetamide (IAA) (Sigma-Aldrich, I61250-10G)
- Sequencing-grade modified Trypsin (Promega, V5111)
- α-Cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, 70990-250MG-F)
- Propylene glycol (PG) (Sigma-Aldrich, W294004-1KG-K)

d. Personnel Required

- One research scientist

5. Procedural guidelines

These procedures are for the preparation of bovine nasal swab samples collected as detailed in the *SOP for oral and nasal swab collection for the diagnosis of bovine tuberculosis (bTB)*, v1.3. Swabs can be either directly analysed using this method, or thawed from quarantine in the -80°C freezer.

Note: It is advisable that all steps will be carried out by keeping the samples and extracts etc. on ice as much as possible. If storage time is needed between the various sample preparation stages, this should be minimized and kept the same within a (disease classification) study. For longer storage times, the samples should be kept in a -80°C freezer.

a. Inactivation and Precipitation

i. Move the swabs to the microbiological safety cabinet (MSC), either from sample quarantine or directly from the transportation packaging.
ii. Inside the MSC, carefully remove swab from its casing and place it into a 1.5-mL tube containing 400µL of 1X PBS, and briefly agitate (at least five times).
iii. Discard swab after squeezing it gently against the inside walls of the 1.5-mL tube.
iv. Add 900µL of 100% ethanol and vortex to mix. Samples should be left on ice if multiple samples are processed at the same time.
v. Centrifuge samples for 5 min at 13,000 rpm. Remove and discard the supernatant and resuspend the resultant pellet in 30µL of 0.1% TFA.

b. Trypsin Digestion

i. Add 50µL of 50mM ammonium bicarbonate to the samples and mix by pipetting.
ii. Add 5µL of 100mM DTT and vortex. Incubate at 37°C for 30 min.
iii. Add 10µL of 100mM IAA and vortex. Incubate at room temperature in the dark for 30 min.
iv. Add 2µL of sequencing grade trypsin (0.2µg/µL) and incubate at 37°C for 2 h.
v. Add 8µL of 10% TFA to inactivate trypsin and stop the reaction.
vi. Purify the samples and extract the peptides by using ZipTips according to the manufacturer's instructions, with a final elution volume of 5µL.

c. Liquid MALDI MS Sample Preparation

i. Prepare a liquid support matrix (LSM) for MALDI analysis by dissolving CHCA in 70:30 ACN:H_2O (%, v/v) at a concentration of 25mg/mL. The solution can be sonicated in an ultrasonic water bath to assist dissolving the CHCA crystals. Once fully dissolved, add PG (by adding 7 volume parts of PG to 10 volume parts of CHCA solution) and mix by vortexing.
ii. For each sample, spot 0.5µL of the LSM on to a stainless steel MALDI sample plate followed by the addition of 0.5µL of the digest sample to the same spot.
iii. Mount the MALDI sample plate on to the plate holder.

d. MS Data Acquisition

Note: The following steps and instrument settings are just for general guidance for liquid AP-MALDI MS analysis of samples as prepared in the previous steps on a Waters Synapt G2-Si instrument.

i. Use the following instrument modes: Mobility TOF, Positive Ion, Sensitivity and MS.
ii. Calibration of the instrument should be performed in Mobility TOF MS mode using sodium iodide over the m/z range of 100-2000.

iii. Set the laser energy to 18µJ/pulse and the laser pulse repetition frequency to 30Hz, continuous pulsing.

Note: The exact threshold laser energy that is best for an effective detection of analyte ion signals depends on the laser focus on the droplet surface and other ion source parameters (e.g. laser beam angle/position on droplet).

iv. Set the ion source to the following settings:
Temperature – 80°C;
Transfer Tube Voltage – 3.0kV;
Cone (counter) gas flow – 180L/h.

v. For data acquisition, use the following settings:
m/z range – 100-2000;
Scan Time – 1 minute;
Scans per Second – 1.

Details about automatic data acquisition and the associated sample stage movement depend on the exact plate size and analytes to be detected, amongst other ion source parameters and analytical objectives. Thus, no further details on automatic data acquisition will be given.

e. Data Analysis

For sample classification, there are many types of software that can be used for statistical modelling. In this case, we have used Abstract Model Builder (AMX, Waters). A training set of LAP-MALDI mass spectral profiles was generated for classification of healthy, mastitis and tuberculosis samples. Cross-validation of the model was performed in the absence of unknown samples to test against the model, whereby 20% of the training set data was left out and tested against the remaining data set.

Example -

i. Start the Abstract Model Builder (AMX) [Beta] version 1.01962.0 software.
ii. Switch to the ‘Sample List’ tab. Click ‘Add sample files’ and select all Waters .raw files to be added to the model, click on the single arrow tab and apply the correct classification to each file according to the sample type (healthy, mastitis, reactor).
iii. Click on ‘Add samples’, which takes you back to the ‘Sample list’ tab.
iv. Select all files and in the ‘TIC Operations’ toolbar, click on ’Spectrum from all scans’.
v. Switch to the ‘Model properties’ tab.
vi. Enter an appropriate model name in the ‘Model name’ field and select ‘LDA model’ from the ‘Model type’ drop-down menu.
vii. In the ‘PCA dimensions’ field, set the value to 10 (or less if you have less .raw files) and in the ‘LDA dimensions’ field, set the value to n-1 (n = number of classes). In the ‘Intensity limit’ field, enter 1.00E2.

viii. Binning is kept at default values, performed every 1 Da in the mass range of 650-2000 in advanced mode.
ix. Under ‘Spectrum interpretation’ select ‘One spectrum per burn’.
x. Under ‘Preprocessing’ only select ‘Apply background subtraction’ and ‘Apply normalization’.
xi. Switch to the ‘Model builder’ tab and click on ‘Build the model’. This can take up to 30 min, if a high number of data files are provided.
xii. Once the model building is complete, switch to the ‘Visualisation’ tab for a representation of the PCA/LDA model. By default, the first 3 components that are most responsible for variation are displayed. This can be altered by clicking the ‘…’ icon on the top toolbar, to the right of the text field, which indicates the displayed components.
xiii. Switch to the ‘Cross validation’ tab. Keep the default settings the same, which are the ‘20% out’ function, with the ‘Outlier’ drop down menu set to ‘Based on standard deviation’ and the ‘Std. dev. multiplier’ set to 5. Enter a suitable report name in the ‘New report name’ field and click on ‘Start’. This can also take up to 30 min.
xiv. Click ‘OK’ when the ‘Cross validation terminated successfully. The report can be seen on the reports page.’ window appears.
xv. Within the ‘Cross validation’ tab, switch to the ‘Reports’ tab. Select the report required, which can be identified by the name and creation time. Selecting the report will display the confusion matrix, the correct classification rate and the identification of outliers.
xvi. The cross validation report can be saved by clicking ‘Save…’ on the appropriate report. By default, this is saved as a .csv file. Click on ‘Save’ and subsequently on ‘OK’ when the ‘CSV file generated successfully.’ window appears.
xvii. To export the data matrix for use in other applications, switch to the ‘Home’ tab and select ‘Export data matrix’ in the top toolbar. This will export the data matrix according to the settings applied in the ‘Model properties’ tab as described in 5.5.6-5.5.10. This is exported as a .csv file.
xviii. When leaving the software, don’t forget to save the current project, following the prompts in the various windows that will appear.

6. References

Ryumin, P., et al. (2016). "Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS." Methods **104**: 11-20.

Supporting Information 4 – Peak List Used for Identifying S100-A12

BEGIN IONS

TITLE=Defra Reactor Protein Combined

PEPMASS=1319.4296

110.07 18749.00

120.08 12778.00

129.10 24703.00

266.16 3624.00

276.15 5837.00

366.18 3410.00

413.21 7690.00

467.22 2207.00

495.23 2389.00

587.30 4037.00

608.31 1324.00

632.33 623.00

724.37 3551.00

794.38 2257.00

1108.09 1728.00

1136.60 4687.00

1249.70 4147.00

3166.82 6347.00

1476.81 7343.00

3352.78 5065.00

1745.95 4596.00

1859.06 12400.00

4757.59 26190.00

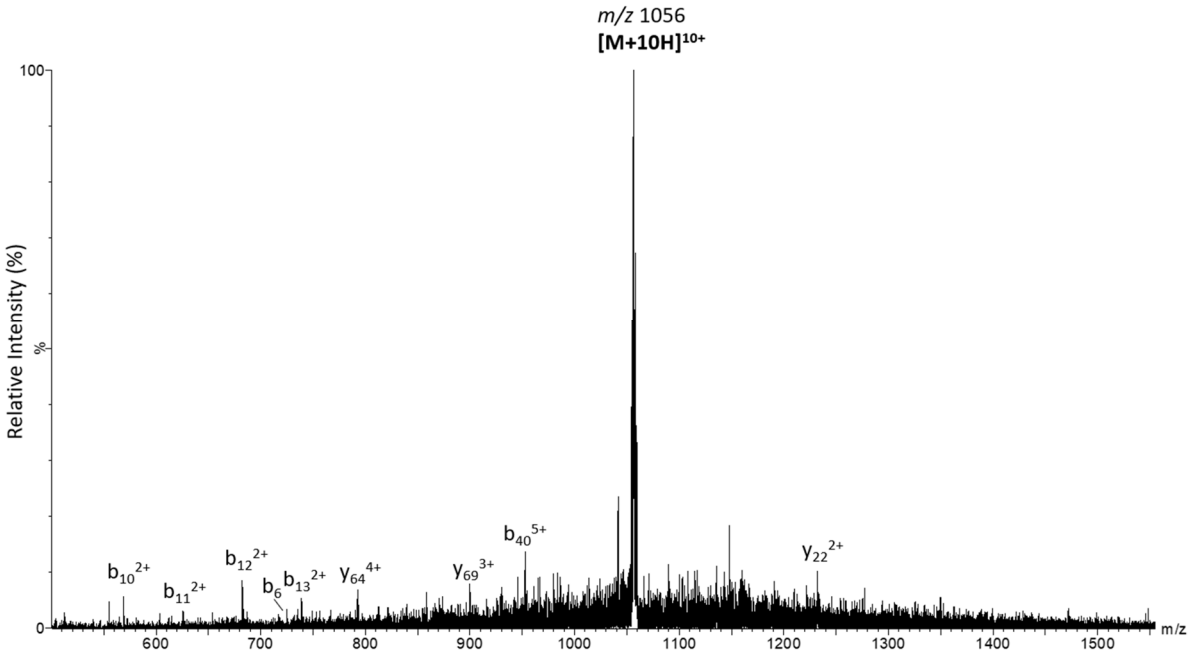
1958.16 24310.00

5910.31 8428.00

4757.63 20180.00

END IONS

Supporting Information 5 – Fragmentation Spectrum of the $[M+10H]^{10+}$ Ion of S100-A12



Chapter 6 - Discussion of Entire Thesis

Development of Methods

Optimisation of the LAP-MALDI set up has been previously detailed for the acquisition of multiply charged ions. This encompasses the use of a heated transfer tube, distance of the mounted sample from the transfer tube inlet, extraction potential difference and application of counter-flow gas. (56) The use of alternative matrices, rather than CHCA as used in this thesis, was also explored. In chapter 3, the use of DHB in figure S2 showed the absence of the detection of multiply charged ions and therefore was not explored further for analysis of proteins. Choice of matrix for LAP-MALDI MS has also been discussed elsewhere previously.(60)

The use of an EtOH/FA extraction for preparation of bacterial samples was initially decided upon due to it's use in clinical MALDI biotyping instruments, where the direct transfer method is not sufficient for an identification. The use of this protocol with clinical biotyping instruments typically produces a proteomic fingerprint of ribosomal proteins, hence it's choice for use to obtain multiply charged protein signals with LAP-MALDI MS. However, it's use in combination with analysis via LAP-MALDI shows the abundant detection of lipid profiles for each bacterium, along with the detection of multiply charged peptide/protein signals. Alternative protocols for simply the acquisition of lipids were also explored, including the Bligh and Dyer method (61) and the Folch method.(62) This was explored to isolate lipids and also remove them in order to focus on protein signals, however this did not yield sufficient data.

A protocol to detect proteins was further developed and is detailed in chapter 4. This protocol involved the use of a clean-up step using C18 ZipTips, and also providing a

concentration effect by using a relatively smaller elution volume which was used for analysis. The use of the ZipTips decreases the relative intensity of lipids analysed, and therefore may contribute to the enhanced detection of proteins. This protocol allows for reproducible detection of several multiply charged signals, from at least one protein from bacteria. The reproducibility allows for efficient MS/MS analysis, which can be used for identification of proteins and contribute to species identification, as demonstrated in figure 2C in chapter 4. The use of low pH conditions for preparation of proteins was compatible with LAP-MALDI MS analysis, in comparison to the use of surfactants which are commonly used in the preparation of biological samples for solubilisation of proteins. These have been investigated with LAP-MALDI MS, showing the presence of cluster formation of the buffer compounds, as well as matrix and salt adducts. The use of surfactants can also affect liquid droplet stability. (63)

The collection of clinical samples for the detection of bTB involved additional challenges for sample preparation. The use of LAP-MALDI MS is sensitive to the presence of polymeric substances such as polyethylene glycol and polypropylene glycol which are components of common consumables used in the laboratory. Various sample collection devices were investigated, including several types of swabs, as well as simply collecting saliva in a sterilin pot. The collection of saliva using a sterilin pot would not have been suitable in terms of health and safety, and the use of saliva as a biofluid did not yield sufficient biomarkers to investigate further. The use of swabs allowed detection of multiply charged ions as well as lower molecular weight biomolecules, presumed to be lipids, and therefore were chosen as the collection method. To avoid detection PEG and PPG signals, wooden handled swabs were chosen to minimise these interferences.

The development of a protocol for the sample preparation of the bovine nasal swabs had the requirement of an initial inactivation stage due to *M. bovis* being a biosafety level (BSL) 3 microorganism. The laboratory where these swabs were handled is category 2 for BSL 2 organisms, and therefore a derogation from this status was obtained from local health and safety authority dependent on immediate activation of these samples. The use of EtOH for inactivation also had a precipitation effect on the biomolecules that were in the samples, which were able to be isolated from the remaining fluids. A continuation of the use of TCA was used with the nasal swabs, followed by a ZipTip clean up in an attempt to produce streamlined protocols for the handling of all clinical samples for analysis via LAP-MALDI MS.

The data summarised in all chapters was subjected to further statistical analysis in order to produce values for correct classification rates. The two methods of statistical modelling included PCA and LDA. These are extremely useful in classifying mass spectrometry data by reducing the dimensionality and determining maximum variance within a data set. The use of PCA is an unsupervised technique, and therefore clustering of species that is performed in chapter 3 is highly specific, as the separation into each species does not take into account any class labels. LDA was applied to the dataset obtained for the exploration with bTB. The use of PCA alone was explored in order to obtain the same success as with the lipid profiles for bacteria, however lacked specificity and sensitivity in terms of identification. An LDA model was therefore used for the bTB data, which produced higher % specificity and sensitivity values. The samples obtained from the nasal swabs were extremely crude in comparison to the bacterial samples which processed from pure cultures, which may contribute to the lack of success with the PCA model.

As the majority of the data used in this thesis was input to a statistical model, the outliers were defined on binned data (every 1 m/z unit). For each model this was defined at 5 standard deviations. For data other than those used in the main statistical models, adequate working standards of the instruments were defined with sufficient intensity and peak shapes. Upon visual inspection of multiply charged ions, the presence of sodium and potassium adducts would indicate poor peak shapes and therefore would not be suitable for fragmentation. This would indicate high salt content from the ion source, the target plate, the LSM or the sample itself. With biological samples, it is difficult to mitigate the presence of salts, and therefore cleaning of the ion source/target plate would be performed, or a new LSM prepared, in order to increase the signal to noise ratio. The total ion count and the intensity of a combined spectrum was also monitored in order to produce sufficient data. For example, a combined spectrum after a one-minute acquisition should have at least 1e5 TIC, a S/N ratio of the desired peaks of 5, and a minimum base peak ion intensity of 8e3, in order to be deemed suitable data.

Context within Wider Research

There is a vital need for clinical diagnostics to provide efficient patient care. Traditional microbiological tests have set the benchmark for identification of infectious disease, such as chromogenic media(64) and biochemical testing.(65) Newer technologies must deliver on ease of use and more rapid results, whilst maintaining high accuracy which can have an impact on the integration of these into good clinical practice guidelines. Clinical MALDI-TOF biotyping was first commercialised by Bruker Daltonik in 2006,(15, 66)given FDA clearance in 2013 (66) and now forms part of the UK standards for microbiology investigations. (67) MALDI-TOF MS biotyping has provided a cost effective, rapid method of microbial diagnostics, where complex mass spectral data is transformed into a simple read-out score

for routine characterisation of microorganisms. Although there are drastically clear benefits to having biotyping MS instruments, there are still some drawbacks to the technique, including limited species databases, as well as the inherent issues of traditional (solid-state) MALDI such as ion flux stability.

The aims of this thesis were to develop LAP-MALDI MS for applications that are highly beneficial to the clinical laboratory, maintaining the benefits conventional MALDI mass spectrometry has already provided, and surpassing these. Initial developments of LAP-MALDI MS enabled the acquisition of multiply charged protein ion signals, which are typically only observed with electrospray ionisation (ESI). (68) ESI is usually coupled to liquid chromatography (LC) systems, adding lengthy separation steps prior to MS analysis, which are not required here. The use of a liquid droplet in comparison to a solid crystalline MALDI sample also overcomes the ion yield stability issue; sample droplets are homogenous and stable for thousands of laser shots (68) and can be stored at 4°C and even -20°C for later analysis.(56) More recently high-throughput (HTP) analysis has been demonstrated with LAP-MALDI MS, whereby intact proteins can be analysed at a rate of up to 10 samples per second.(69)

The acquisition of lipids using LAP-MALDI MS is extremely straightforward. From the initial experimental studies using standards in negative ion mode,(70) where lipids are typically more plentiful, the production of lipid profiles from biological fluids (caprine and ovine milk) was quickly shown and part of LAP-MALDI's application in livestock speciation and veterinary diagnostics.(71, 72) Lipid profiling by LAP-MALDI MS was further developed in chapter 3, acquiring unique lipid profiles for clinically relevant bacteria allowing species differentiation using unsupervised principal component analysis.

The analysis of lipids is often dismissed with the 'soft' ionisation techniques of MALDI and ESI. Electrospray ionisation is not compatible with the use of non-polar solvents; since many lipids are non-polar ESI is not typically the method of choice. The use of MALDI for lipid analysis is also not favoured due to the presence of matrix ion peaks, and often overlapping contaminants, in the m/z range below 1,500 where lipid signals are typically recorded. Historically, lipid profiling of bacteria was performed with MS techniques including pyrolysis(73) and fast atom bombardment.(74) In the last two decades this has been overtaken by many ambient ionisation techniques.

In this thesis it has been demonstrated that LAP-MALDI MS offers several advantages for bacterial classification over current ambient and biotyping methodologies. Sample preparation is simple and minimal, which is a requirement for a robust method that can be readily employed by clinical laboratory technicians. All lipid profiles with LAP-MALDI MS were acquired in positive ion mode, where other ionisation techniques such as paperspray ionisation require both positive and negative ion mode for identification(75).Correct classification of bacteria to 98.63% was achieved with LAP-MALDI MS, in comparison to REIMS where classification was only performed to 95.9%(39).

With the focus primarily remaining on the detection of small organic molecules, chapter 4 discusses the application of LAP-MALDI to a dual-assay incorporating multiplex detection of antibiotic resistance, as well as species identification of bacteria. β-lactam antibiotics inhibit the activity of bacterial transpeptidases enzymes, which inhibit the formation of their cell walls and therefore possess bactericidal activity.(76) Penicillin is famously the first antibiotic discovered in 1942,(77) and since then β-lactam antibiotics encompass many different classes including penicillins, cephalosporins and carbapenems. Selective pressure applied by

the use of antibiotics has led to the evolution of bacterial enzymes conferring resistance to antibiotics.(78) In addition, pressures have been applied from agriculture and aquatic systems, whereby bacteria and antibiotics have been exchanged between ecosystems via direct spread, urine/faeces or foodstuffs.(79) There are also social issues which contribute to increased antibiotic use, and in turn resistance, including ageing populations. In lower economically developed countries, the lack of clean water and poor sanitation allows spread of bacteria, potentially harbouring resistance.(80)

Antibiotic resistance incurs a high cost and high mortality rate, and early diagnosis is often insufficient in many healthcare institutions.(81) Early diagnosis could minimise indirect effects of resistant outbreaks, such as ward closures and control measures in hospitals which could have an impact on uninfected patients. Ward closures and other control measures provide additional costs and prolong patient treatment. Current UK guidelines for antimicrobial susceptibility testing relies on phenotypic culture methods, either via selective media or enrichment broths.(82) Molecular methods are only considered in serious cases where rapid results are required. There is therefore a clear need for a new technology for reliable and rapid AMR testing.

In the case of β-lactams, it is β-lactamase enzymes that hydrolyse the β-lactam ring rendering it ineffective against treatment of bacterial infections. Hydrolysis of antibiotics by bacteria can be readily detected using mass spectrometry, and commercial kits have been developed to assess resistance to a single reference antibiotic, in contrast to the work discussed in chapter 4, whereby a panel of different classes of antibiotics were tested against. The hydrolysis of antibiotics is a basic assay which most instruments are capable of detecting; many MALDI-TOF based research articles have been published to show this.(83,

84) The instruments typically used for these experiments are axial-TOF instruments which have high sensitivity, but lower resolution and accuracy than hybrid QTOFs as used in this thesis. Resolution and accuracy are imperative for MALDI-based assays to be able to distinguish between the regularly discussed CHCA matrix ion cluster peaks and antibiotic peaks. The additional benefit of using a QTOF instrument and therefore MS/MS analysis in this case would allow for a quality control step to fragment suspected antibiotic-related peaks to confirm their identity, rather than simply mass matching. MS/MS analysis using alternative methods poses issues that are not encountered by LAP-MALDI MS. For instance, MALDI-based fragmentation has lower ion signal stability due to inhomogeneous crystalline sample spots and is typically poor if performed on axial-TOF instruments using post-source decay (PSD), and ESI MS/MS is typically coupled to lengthy chromatography separation. Using the LAP-MALDI MS assay and the same sample droplet as employed for resistance detection and species identification, controlled fragmentation can be applied for structural elucidation of ion signals within 1-2 min of additional analysis time.

A strain of *K. pneumoniae* used in this study possessed OXA-48 β-lactamase, a class D lactamase with a known strong resistance to penicillins, and weak activity against cephalosporins.(85) This is supported by the data presented in chapter 4. This strain also showed weak activity against meropenem, however outbreaks of carbapenem resistance have recently been reported.(86, 87) This assay would be able to detect newly acquired resistance for multiple classes of antibiotics. *K. pneumoniae* is deemed one of the most concerning pathogens in terms of antibiotics resistance, being part of the ESKAPE group of organisms which pose the biggest threat to human health.(88) Infections of *K. pneumoniae* account for approximately one third of Gram-negative infections, and rates of antibiotic resistance have steadily increased since 2005 in this species.(89) The possession of antibiotic

resistance genes results in patient treatments initiated approximately 72 h following suspected infection, in comparison to 11.5 h for non-resistant strains.(90) Administration of broad-spectrum antibiotics to patients with suspected resistant infections is not sustainable for public health, and therefore accurate diagnosis of specific resistance is imperative.

In addition to the multiplex detection of antibiotics, the assay developed in chapter 4 retains the acquisition of bacterial lipids in the LAP-MALDI MS spectrum. The reduction in time from not using two separate tests for identification and AMR detection will significantly increase the throughput of samples to be analysed, and therefore will allow more rapid initiation of patient treatment. Conventional MALDI-TOF MS has been shown to detect colistin resistance in bacteria, simply from glycolipid profiles.(91) However, the limit of detection for the glycolipids is much higher than with current diagnostics, and this assay is currently only applicable for the detection of a single class of antibiotics, in comparison to the LAP-MALDI MS assay where several were included.

As antibiotics have different mechanisms of action, not all resistance mechanisms will be detected via β-lactamase-mediated hydrolysis. Therefore, a combination of assays will still have to be employed to account for different resistance genes. As LAP-MALDI MS has the capability to easily detect lipids, the alteration of lipid profiles due to specific resistance genes can be investigated further in the future.

Acquisition of both lipids and protein ion signal was performed in chapter 3 and 4 for bacterial extracts for the purpose of bacterial identification via profiling. This is highly beneficial for identification purposes to use additional biomarkers, rather than simply proteins for identification via profiling. The identification of certain resistance mechanisms has been performed via sequencing of specific biomarkers that are implicated in resistance.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) have commonly been distinguished using mass spectrometry by the analysis of penicillin-binding protein (PBP2a). PBP2a has a low binding affinity for methicillin, therefore conferring resistance.(92) PBP2a also has low affinity for β-lactams, and can therefore be investigated for β-lactam resistance.(93) Many studies have profiled the presence of PBP2a using traditional MALDI, however the direct identification had to be confirmed by alternative methods such as N-term sequencing and immunogenic assays.(94) Lack of reproducibility of MALDI-TOF identification of MRSA has also been reported.(95) Top-down identification of the PBP2a protein has been performed using a short (5 minute) LC gradient from recombinant protein.(96) However, it has not yet been directly performed on clinically relevant samples, let alone directly using MALDI. The use of LAP-MALDI MS has the potential to provide the direct identification of these proteins and their antibiotic resistance, without the need for chromatographic separation or alternative techniques. (96)

The field of diagnostics is also highly valuable for animal health. Whilst maintaining the benefits of a valuable diagnostic test, animal and veterinary diagnostic tests should also be extremely simple to perform in the field. Sample collection from animals is not as straightforward as for humans, and therefore a non-invasive sample collection is ideal. Animal samples should also be easy and safe to collect to minimise danger to humans. MALDI MS biotyping has its place in veterinary diagnostics, but is not routinely performed and therefore databases may not be sufficiently populated. However, many bacteria causing bovine mastitis are also human pathogens and are therefore present in these databases.(97) Nonetheless, the time from microbial sample collection to travel to the laboratory and subsequent culture time is not ideal for veterinary workflows. Rapid lateral flow tests have been commercialised by veterinary pharmaceutical companies such as Zoetis and IDEXX,

however these require a blood sample and is therefore relatively invasive and is only targeted to specific organisms.(98) Other veterinary pathogens can take days to weeks for culture, such as many *Mycobacterium* species, and therefore would not be suitable for large-scale screening that often takes place to detect these infections.(99)

Chapter 5 discusses the application of LAP-MALDI MS to the diagnosis of bovine tuberculosis, caused by *M. bovis*. Due to the life cycle of *M. bovis,* in particular the stage of infection to various host animals, eradication of the bacterium is extremely difficult, therefore several control measures for bTB have been previously investigated such as environmental controls,(100) as well as vaccines.(101) . The current diagnostics of tuberculin skin testing and IFNy testing detect an inflammatory response in the animal;(102, 103) *M. bovis* infection is often latent, and therefore the numbers of mycobacterial cells circulating the body are often low.(104) In the UK, bTB requires country wide screening, therefore culturing cells is not a viable option.(105)

For a potential LAP-MALDI-based bTB test, the proposed sample preparation utilises nasal fluids that are collected using cotton tipped swabs. These are far less invasive than the collection of blood, or the injection of tuberculin into animals. Lateral flow antigen tests have been developed for the detection of bTB, however these are not effective with the use of non-invasively collected specimens such as milk and urine.(106) The total LAP-MALDI sample preparation time from sample receipt in the laboratory to result was under 4 h, far reducing the time for the tuberculin test which is the current approved test for bTB screening.(107)

It was expected with LAP-MALDI MS that proteinaceous material from the mycobacterium itself was not going to be detected, but rather a response from the animal. The detection of

S100-A12, an inflammatory protein, highly correlated with infection in bTB-infected animals. The use of bovine mastitis was used as an infection positive control, to determine whether the discrimination between bTB and healthy samples was bacterium specific or just a response to infection. Application of a 3-class model shows that the detection of bTB using LAP-MALDI MS has a sensitivity and specificity of 75.0% and 90.1% respectively. S100-A12 is released in response to infection, and therefore is also present in mastitis infection samples. The use of simply S100-A12 however, could be used in a triage screening system, whereby diseased animals are identified, and then subjected to further diagnostic tests, rather than the large scale, relatively more invasive bTB testing that is currently undertaken.

The sample preparation for this LAP-MALDI-based bovine disease assay involved a short enzymatic digestion. Despite the digestion step S100-A12 is detected as a full-length protein. Other diagnostic biomarkers for bTB may be present in the samples, requiring further identification. The identification of S100-A12 was performed via protein sequencing using top-down LAP-MALDI MS/MS analysis. The identification of proteins from bovine samples is virtually impossible without protein sequencing, as unlike pure bacterial cultures, the nasal swabs collected will have possessed many interfering substances, and therefore even with high mass accuracy the identification would not confidently be performed. The ability to perform top-down MS/MS from a 0.5-µL droplet is a key benefit of LAP-MALDI, where lengthy chromatography steps and high samples volumes are not required, which has been demonstrated for various applications in this thesis. In clinical settings, a proposed workflow is the use of an initial LAP-MALDI MS profile using lipidomic and proteomic biomarkers for high confidence diagnostics. Where ambiguous identifications are obtained, or more definitive identification is required, perhaps where novel biomarkers are observed, MS/MS can be performed for sequencing purposes.

Chapter 7 - Conclusions and Future Perspectives

The potential value of LAP-MALDI MS for the clinical laboratory has been shown in this thesis, requiring minimal reagents and providing rapid results. In each chapter the applicability of LAP-MALDI MS for the analysis of clinical, both human and animal, has been demonstrated. These proof-of-principle studies can be further explored in many ways. Firstly, high-throughput analysis could be performed. Many studies using LAP-MALDI for high-throughput analysis have been published, most recently including the detection of intact proteins rather than simply smaller organic molecules and peptides.

Following the success of bacterial lipid profiling, proteins from clinical bacterial samples could be profiled, further enhancing the benefits of LAP-MALDI MS in a clinical laboratory. For the analysis of bacteria, one strain per species was analysed in this thesis. Multiple strains per species should be analysed using LAP-MALDI, firstly to determine whether differences in lipidomic and proteomic profiles can be observed, but also for subsequent sequencing of proteins. If there are no observable differences in the profiles, protein sequencing by MS/MS may reveal subtle differences in the amino acid sequence to allow for sub-species identification.

Sequencing of proteins would also be beneficial for AMR detection; detection of hydrolysis of antibiotics due to resistance enzymes is only sufficient for β-lactam resistance. Alternative classes of antibiotics such as aminoglycosides and tetracyclines have different mechanisms of action and therefore their detection would only be sufficient by the sequencing of resistance-specific biomarkers, whether these be novel proteins or mutations in existing ones. Further investigation of AMR could be performed with lipids in terms of the identification of lipid A, which is widely documented to be implicated in AMR. (108)

Investigation of lipid profiles should also be performed in negative ion mode, which is commonly used in lipidomics but was not explored in this thesis.

The sample preparation for bacterial samples could also be further investigated. The direct transfer method is often used with commercial biotyping instruments, and it is claimed that the application of the acidic matrix is sufficient for effective ribosomal protein extraction. An acidic environment is provided in liquid samples, as well as the application of the laser which could provide enough energy for cell lysis and direct analysis of lipids and proteins. This has not yet been explored due to health and safety limitations.

References for Chapters 1, 2, 6 and 7

1. Boleij A, Tjalsma H. Gut bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. Biological Reviews. 2012;87(3):701-30.
2. NHS (2021) *Hospital Admitted Patient Care Activity 2020-21*. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/hospital-admitted-patient-care-activity/2020-21 (Accessed 13 March 2023)
3. Gregersen T. Rapid method for distinction of gram-negative from gram-positive bacteria. European Journal of Applied Microbiology and Biotechnology. 1978;5(2):123-7.
4. Facklam R, Elliott JA. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clinical Microbiology Reviews. 1995;8(4):479-95.
5. Koser SA. Utilization of the Salts of Organic Acids by the Colon-Aerogenes Group. Journal of Bacteriology. 1923;8(5):493-520.
6. Gao F, Melody T, Daniels DF, Giles S, Fox S. The impact of compliance with 6-hour and 24-hour sepsis bundles on hospital mortality in patients with severe sepsis: a prospective observational study. Critical Care. 2005;9(6):R764.
7. Ebner K, Suda M, Watzinger F, Lion T. Molecular Detection and Quantitative Analysis of the Entire Spectrum of Human Adenoviruses by a Two-Reaction Real-Time PCR Assay. Journal of Clinical Microbiology. 2005;43(7):3049-53.
8. Timms JF, Hale OJ, Cramer R. Advances in mass spectrometry-based cancer research and analysis: from cancer proteomics to clinical diagnostics. Expert Review of Proteomics. 2016;13(6):593-607.
9. Hou T-Y, Chiang-Ni C, Teng S-H. Current status of MALDI-TOF mass spectrometry in clinical microbiology. Journal of Food and Drug Analysis. 2019;27(2):404-14.
10. Hill WE, Wachsmuth K. The polymerase chain reaction: Applications for the detection of foodborne pathogens. Critical Reviews in Food Science and Nutrition. 1996;36(1-2):123-73.
11. Chau CH, Strope JD, Figg WD. COVID-19 Clinical Diagnostics and Testing Technology. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2020;40(8):857-68.
12. Surkova E, Nikolayevskyy V, Drobniewski F. False-positive COVID-19 results: hidden problems and costs. The Lancet Respiratory Medicine. 2020;8(12):1167-8.
13. Anhalt JP, Fenselau C. Identification of bacteria using mass spectrometry. Analytical Chemistry. 1975;47(2):219-25.
14. Singhal N, Kumar M, Kanaujia PK, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Frontiers in Microbiology. 2015;6.
15. Maier T, Klepel S, Renner U, Kostrzewa M. Fast and reliable MALDI-TOF MS–based microorganism identification. Nature Methods. 2006;3(4):i-ii.
16. Sogawa K, Watanabe M, Sato K, Segawa S, Ishii C, Miyabe A, et al. Use of the MALDI BioTyper system with MALDI-TOF mass spectrometry for rapid identification of microorganisms. Analytical and Bioanalytical Chemistry. 2011;400(7):1905-11.
17. Lévesque S, Dufresne PJ, Soualhine H, Domingo MC, Bekal S, Lefebvre B, et al. A Side by Side Comparison of Bruker Biotyper and VITEK MS: Utility of MALDI-TOF MS Technology for Microorganism Identification in a Public Health Reference Laboratory. PLoS One. 2015;10(12):e0144878.
18. Marko DC, Saffert RT, Cunningham SA, Hyman J, Walsh J, Arbefeville S, et al. Evaluation of the Bruker Biotyper and Vitek MS Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry Systems for Identification of Nonfermenting Gram-Negative Bacilli Isolated from Cultures from Cystic Fibrosis Patients. Journal of Clinical Microbiology. 2012;50(6):2034-9.
19. van den Beld MJC, Rossen JWA, Evers N, Kooistra-Smid MAMD, Reubsaet FAG. MALDI-TOF MS Using a Custom-Made Database, Biomarker Assignment, or Mathematical Classifiers Does Not Differentiate *Shigella* spp. and *Escherichia coli*. Microorganisms. 2022;10(2):435.

20. Pavlovic M, Konrad R, Iwobi AN, Sing A, Busch U, Huber I. A dual approach employing MALDI-TOF MS and real-time PCR for fast species identification within the *Enterobacter cloacae* complex. FEMS Microbiology Letters. 2012;328(1):46-53.
21. Cunningham SA, Mainella JM, Patel R. Misidentification of Neisseria polysaccharea as Neisseria meningitidis with the Use of Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry. Journal of Clinical Microbiology. 2014;52(6):2270-1.
22. He Y, Li H, Lu X, Stratton CW, Tang Y-W. Mass Spectrometry Biotyper System Identifies Enteric Bacterial Pathogens Directly from Colonies Grown on Selective Stool Culture Media. Journal of Clinical Microbiology. 2010;48(11):3888-92.
23. Yo CH, Shen YH, Hsu WT, Mekary RA, Chen ZR, Lee WJ, et al. MALDI-TOF mass spectrometry rapid pathogen identification and outcomes of patients with bloodstream infection: A systematic review and meta-analysis. Microbial Biotechnology. 2022;15(10):2667-82.
24. Tran A, Alby K, Kerr A, Jones M, Gilligan PH. Cost Savings Realized by Implementation of Routine Microbiological Identification by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology. 2015;53(8):2473-9.
25. Lagacé-Wiens PR, Adam HJ, Karlowsky JA, Nichol KA, Pang PF, Guenther J, et al. Identification of blood culture isolates directly from positive blood cultures by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and a commercial extraction system: analysis of performance, cost, and turnaround time. Journal of Clinical Microbiology. 2012;50(10):3324-8.
26. Randall LP, Lemma F, Koylass M, Rogers J, Ayling RD, Worth D, et al. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. Research in Veterinary Science. 2015;101:42-9.
27. Cassagne C, Normand A-C, L'Ollivier C, Ranque S, Piarroux R. Performance of MALDI-TOF MS platforms for fungal identification. Mycoses. 2016;59(11):678-90.
28. Wolk DM, Kaleta EJ, Wysocki VH. PCR–Electrospray Ionization Mass Spectrometry: The Potential to Change Infectious Disease Diagnostics in Clinical and Public Health Laboratories. The Journal of Molecular Diagnostics. 2012;14(4):295-304.
29. Özenci V, Patel R, Ullberg M, Strålin K. Demise of Polymerase Chain Reaction/Electrospray Ionization-Mass Spectrometry as an Infectious Diseases Diagnostic Tool. Clinical Infectious Diseases. 2017;66(3):452-5.
30. Catherman AD, Skinner OS, Kelleher NL. Top Down proteomics: facts and perspectives. Biochemical and Biophysical Research Communications. 2014;445(4):683-93.
31. Cramer R, Pirkl A, Hillenkamp F, Dreisewerd K. Liquid AP-UV-MALDI enables stable ion yields of multiply charged peptide and protein ions for sensitive analysis by mass spectrometry. Angewandte Chemie International Edition. 2013;52(8):2364-7.
32. Smith PBW, Snyder AP, Harden CS. Characterization of Bacterial Phospholipids by Electrospray Ionization Tandem Mass Spectrometry. Analytical Chemistry. 1995;67(11):1824-30.
33. Cody RB, McAlpin CR, Cox CR, Jensen KR, Voorhees KJ. Identification of bacteria by fatty acid profiling with direct analysis in real time mass spectrometry. Rapid Communications in Mass Spectrometry. 2015;29(21):2007-12.
34. Zhang JI, Talaty N, Costa AB, Xia Y, Tao WA, Bell R, et al. Rapid direct lipid profiling of bacteria using desorption electrospray ionization mass spectrometry. International Journal of Mass Spectrometry. 2011;301(1):37-44.
35. Cameron SJS, Bodai Z, Temelkuran B, Perdones-Montero A, Bolt F, Burke A, et al. Utilisation of Ambient Laser Desorption Ionisation Mass Spectrometry (ALDI-MS) Improves Lipid-Based Microbial Species Level Identification. Scientific Reports. 2019;9(1):3006.
36. Schäfer K-C, Dénes J, Albrecht K, Szaniszló T, Balog J, Skoumal R, et al. In Vivo, In Situ Tissue Analysis Using Rapid Evaporative Ionization Mass Spectrometry. Angewandte Chemie International Edition. 2009;48(44):8240-2.

37. Kosek V, Uttl L, Jírů M, Black C, Chevallier O, Tomaniová M, et al. Ambient mass spectrometry based on REIMS for the rapid detection of adulteration of minced meats by the use of a range of additives. Food Control. 2019;104:50-6.
38. Robson K, Birse N, Chevallier O, Elliott C. Metabolomic profiling to detect different forms of beef fraud using rapid evaporative ionisation mass spectrometry (REIMS). npj Science of Food. 2022;6(1):9.
39. Strittmatter N, Rebec M, Jones EA, Golf O, Abdolrasouli A, Balog J, et al. Characterization and Identification of Clinically Relevant Microorganisms Using Rapid Evaporative Ionization Mass Spectrometry. Analytical Chemistry. 2014;86(13):6555-62.
40. Bodai Z, Cameron S, Bolt F, Simon D, Schaffer R, Karancsi T, et al. Effect of Electrode Geometry on the Classification Performance of Rapid Evaporative Ionization Mass Spectrometric (REIMS) Bacterial Identification. Journal of the American Society for Mass Spectrometry. 2018;29(1):26-33.
41. Bolt F, Cameron SJS, Karancsi T, Simon D, Schaffer R, Rickards T, et al. Automated High-Throughput Identification and Characterization of Clinically Important Bacteria and Fungi using Rapid Evaporative Ionization Mass Spectrometry. Analytical Chemistry. 2016;88(19):9419-26.
42. Cai HY, Caswell JL, Prescott JF. Nonculture molecular techniques for diagnosis of bacterial disease in animals: a diagnostic laboratory perspective. Veterinary Pathology. 2014;51(2):341-50.
43. Piras C, Hale OJ, Reynolds CK, Jones AB, Taylor N, Morris M, et al. LAP-MALDI MS coupled with machine learning: an ambient mass spectrometry approach for high-throughput diagnostics. Chemical Science. 2022;13(6):1746-58.
44. Barreiro JR, Gonçalves JL, Braga PAC, Dibbern AG, Eberlin MN, Veiga dos Santos M. Non-culture-based identification of mastitis-causing bacteria by MALDI-TOF mass spectrometry. Journal of Dairy Science. 2017;100(4):2928-34.
45. Bacanelli G, Olarte LC, Silva MR, Rodrigues RA, Carneiro PAM, Kaneene JB, et al. Matrix Assisted Laser Desorption Ionization-Time-of-Flight mass spectrometry identification of *Mycobacterium bovis* in Bovinae. Journal of Veterinary Medical Science. 2019;81(10):1400-8.
46. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022;399(10325):629-55.
47. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, et al. Antimicrobial Resistance in ESKAPE Pathogens. Clinical Microbiology Reviews. 2020;33(3).
48. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016;6(2):71-9.
49. Richter SS, Ferraro MJ. Susceptibility Testing Instrumentation and Computerized Expert Systems for Data Analysis and Interpretation. Manual of Clinical Microbiology2011. p. 1144-54.
50. Ligozzi M, Bernini C, Bonora MG, Fatima Md, Zuliani J, Fontana R. Evaluation of the VITEK 2 System for Identification and Antimicrobial Susceptibility Testing of Medically Relevant Gram-Positive Cocci. Journal of Clinical Microbiology. 2002;40(5):1681-6.
51. Pournajaf A, Ardebili A, Goudarzi L, Khodabandeh M, Narimani T, Abbaszadeh H. PCR-based identification of methicillin–resistant *Staphylococcus aureus* strains and their antibiotic resistance profiles. Asian Pacific Journal of Tropical Biomedicine. 2014;4:S293-S7.
52. Váradi L, Luo JL, Hibbs DE, Perry JD, Anderson RJ, Orenga S, et al. Methods for the detection and identification of pathogenic bacteria: past, present, and future. Chemical Society Reviews. 2017;46(16):4818-32.
53. Anantharajah A, Tossens B, Olive N, Kabamba-Mukadi B, Rodriguez-Villalobos H, Verroken A. Performance Evaluation of the MBT STAR®-Carba IVD Assay for the Detection of Carbapenemases With MALDI-TOF MS. Frontiers in Microbiology. 2019;10.
54. Torres-Corral Y, Santos Y. Predicting antimicrobial resistance of Lactococcus garvieae: PCR detection of resistance genes versus MALDI-TOF protein profiling. Aquaculture. 2022;553:738098.

55. Hale OJ, Morris M, Jones B, Reynolds CK, Cramer R. Liquid Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Adds Enhanced Functionalities to MALDI MS Profiling for Disease Diagnostics. ACS Omega. 2019;4(7):12759-65.
56. Ryumin P, Brown J, Morris M, Cramer R. Investigation and optimization of parameters affecting the multiply charged ion yield in AP-MALDI MS. Methods. 2016;104:11-20.
57. Tholey A, Heinzle E. Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry—applications and perspectives. Analytical and Bioanalytical Chemistry. 2006;386(1):24-37.
58. Smirnov IP, Zhu X, Taylor T, Huang Y, Ross P, Papayanopoulos IA, et al. Suppression of alpha-cyano-4-hydroxycinnamic acid matrix clusters and reduction of chemical noise in MALDI-TOF mass spectrometry. Analytical Chemistry. 2004;76(10):2958-65.
59. Lellman SE, Cramer R. Bacterial identification by lipid profiling using liquid atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Clinical Chemistry and Laboratory Medicine. 2020;58(6):930-8.
60. Towers, M.W., Cramer, R. Ionic Liquids and Other Liquid Matrices for Sensitive MALDI MS Analysis. In: Cramer, R. Advances in MALDI and Laser-Induced Soft Ionization Mass Spectrometry. Springer, 2016. P51.
61. Bligh ED, Dyer WJ. A Rapid Method of Total Lipid Extraction and Purification. Canadian Journal of Biochemistry and Physiology. 1959;37(8):911-917.
62. Folch J, Lees M, Stanley GHS. A Simple Method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry. 1957;226:497-509.
63. Krenkel H, Brown J, Morris M, Cramer R. The use of salts, buffers and surfactants in LAP-MALDI MS. International Journal of Mass Spectrometry. 2023;493:117134.
64. Perry JD, Freydière AM. The application of chromogenic media in clinical microbiology. Journal of Applied Microbiology. 2007;103(6):2046-55.
65. Altheide ST. Biochemical and Culture-Based Approaches to Identification in the Diagnostic Microbiology Laboratory. American Society for Clinical Laboratory Science. 2020:ascls.119.001875.
66. Bruker (2013) *Corporation Announces FDA clearance to Market the MALDI Biotyper CA system*. Available from: https://ir.bruker.com/press-releases/press-release-details/2013/Bruker-Corporation-Announces-FDA-Clearance-to-Market-the-MALDI-Biotyper-CA-System/default.aspx. (Accessed 10 May 2023).
67. Public Health England. (2019). Matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF MS) test . UK Standards for Microbiology Investigations. TP 40 Issue 1.1. https://www.gov.uk/uk-standards-for-microbiologyinvestigations-smi-quality-and-consistency-in-clinical-laboratories
68. Cramer R, Pirkl A, Hillenkamp F, Dreisewerd K. Liquid AP-UV-MALDI Enables Stable Ion Yields of Multiply Charged Peptide and Protein Ions for Sensitive Analysis by Mass Spectrometry. Angewandte Chemie International Edition. 2013;52(8):2364-7.
69. Challen B, Morris M, Cramer R. Ultra-High-Throughput and Low-Volume Analysis of Intact Proteins with LAP-MALDI MS. Journal of the American Society for Mass Spectrometry. 2023.
70. Hale OJ, Cramer R. Collision-induced dissociation of doubly-charged barium-cationized lipids generated from liquid samples by atmospheric pressure matrix-assisted laser desorption/ionization provides structurally diagnostic product ions. Analytical and Bioanalytical Chemistry. 2018;410(5):1435-44.
71. Piras C, Ceniti C, Hartmane E, Costanzo N, Morittu VM, Roncada P, et al. Rapid Liquid AP-MALDI MS Profiling of Lipids and Proteins from Goat and Sheep Milk for Speciation and Colostrum Analysis. Proteomes. 2020;8(3):20.
72. Piras C, Hale OJ, Reynolds CK, Jones AK, Taylor N, Morris M, et al. LAP-MALDI MS coupled with machine learning: an ambient mass spectrometry approach for high-throughput diagnostics. Chemical Science. 2022;13(6):1746-58.

73. Basile F, Beverly MB, Voorhees KJ, Hadfield TL. Pathogenic bacteria: their detection and differentiation by rapid lipid profiling with pyrolysis mass spectrometry. TrAC Trends in Analytical Chemistry. 1998;17(2):95-109.
74. Heller DN, Cotter RJ, Fenselau C, Uy OM. Profiling of bacteria by fast atom bombardment mass spectrometry. Analytical Chemistry. 1987;59(23):2806-9.
75. Hamid AM, Jarmusch AK, Pirro V, Pincus DH, Clay BG, Gervasi G, et al. Rapid Discrimination of Bacteria by Paper Spray Mass Spectrometry. Analytical Chemistry. 2014;86(15):7500-7.
76. Oates JAMD, Wood AJJMD, Donowitz GRMD, Mandell GLMD. Drug Therapy: Beta-Lactam Antibiotics. The New England Journal of Medicine. 1988;318(7):419-26.
77. Gaynes R. The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use. Emerging Infectious Diseases. 2017;23(5):849-53.
78. Kolář M, Urbánek K, Látal T. Antibiotic selective pressure and development of bacterial resistance. International Journal of Antimicrobial Agents. 2001;17(5):357-63.
79. Andersson DI, Balaban NQ, Baquero F, Courvalin P, Glaser P, Gophna U, et al. Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiology Reviews. 2020;44(2):171-88.
80. Bartley PS, Domitrovic TN, Moretto VT, Santos CS, Ponce-Terashima R, Reis MG, et al. Antibiotic Resistance in Enterobacteriaceae from Surface Waters in Urban Brazil Highlights the Risks of Poor Sanitation. American Journal of Tropical Medicine and Hygiene. 2019;100(6):1369-77.
81. Akova M. Epidemiology of antimicrobial resistance in bloodstream infections. Virulence. 2016;7(3):252-66.
82. Public Health England. (2020) Investigation of specimens for screening for MRSA. UK Standards for Microbiology Investigations. B 9 Issue 7. https://www.gov.uk/uk-standards-for-microbiologyinvestigations-smi-quality-and-consistency-in-clinical-laboratories
83. Hooff GP, van Kampen JJA, Meesters RJW, van Belkum A, Goessens WHF, Luider TM. Characterization of β-Lactamase Enzyme Activity in Bacterial Lysates using MALDI-Mass Spectrometry. Journal of Proteome Research. 2012;11(1):79-84.
84. Ghebremedhin B, Halstenbach A, Smiljanic M, Kaase M, Ahmad-Nejad P. MALDI-TOF MS based carbapenemase detection from culture isolates and from positive blood culture vials. Annals of Clinical Microbiology and Antimicrobials. 2016;15(1):5.
85. Poirel L, Potron A, Nordmann P. OXA-48-like carbapenemases: the phantom menace. Journal of Antimicrobial Chemotherapy. 2012;67(7):1597-606.
86. Mathers AJ, Hazen KC, Carroll J, Yeh AJ, Cox HL, Bonomo RA, et al. First Clinical Cases of OXA-48-Producing Carbapenem-Resistant *Klebsiella pneumoniae* in the United States: the Menace Arrives in the New World. Journal of Clinical Microbiology. 2013;51(2):680-3.
87. Kola A, Piening B, Pape U-F, Veltzke-Schlieker W, Kaase M, Geffers C, et al. An outbreak of carbapenem-resistant OXA-48 – producing *Klebsiella pneumonia* associated to duodenoscopy. Antimicrobial Resistance and Infection Control. 2015;4(1):8.
88. Oliveira DMPD, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, et al. Antimicrobial Resistance in ESKAPE Pathogens. Clinical Microbiology Reviews. 2020;33(3):e00181-19.
89. Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. FEMS Microbiology Reviews. 2017;41(3):252-75.
90. Friedman ND, Temkin E, Carmeli Y. The negative impact of antibiotic resistance. Clinical Microbiology and Infection. 2016;22(5):416-22.
91. Leung LM, Fondrie WE, Doi Y, Johnson JK, Strickland DK, Ernst RK, et al. Identification of the ESKAPE pathogens by mass spectrometric analysis of microbial membrane glycolipids. Scientific Reports. 2017;7(1):6403.
92. Shalaby MW, Dokla EME, Serya RAT, Abouzid KAM. Penicillin binding protein 2a: An overview and a medicinal chemistry perspective. European Journal of Medicinal Chemistry. 2020;199:112312.

93. Zapun A, Contreras-Martel C, Vernet T. Penicillin-binding proteins and β-lactam resistance. FEMS Microbiology Reviews. 2008;32(2):361-85.
94. Sogawa K, Watanabe M, Ishige T, Segawa S, Miyabe A, Murata S, et al. Rapid Discrimination between Methicillin-Sensitive and Methicillin-Resistant Staphylococcus aureus Using MALDI-TOF Mass Spectrometry. Biocontrol Science. 2017;22(3):163-9.
95. Paskova V, Chudejova K, Sramkova A, Kraftova L, Jakubu V, Petinaki EA, et al. Insufficient repeatability and reproducibility of MALDI-TOF MS-based identification of MRSA. Folia Microbiologica. 2020;65(5):895-900.
96. Neil JR, Verma A, Kronewitter SR, McGee WM, Mullen C, Viirtola M, et al. Rapid MRSA detection via tandem mass spectrometry of the intact 80 kDa PBP2a resistance protein. Scientific Reports. 2021;11(1):18309.
97. Nonnemann B, Lyhs U, Svennesen L, Kristensen KA, Klaas IC, Pedersen K. Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. Journal of Dairy Science. 2019;102(3):2515-24.
98. Levy JK, Crawford PC, Tucker SJ. Performance of 4 Point-of-Care Screening Tests for Feline Leukemia Virus and Feline Immunodeficiency Virus. Journal of Veterinary Internal Medicine. 2017;31(2):521-6.
99. Soini H, Musser JM. Molecular Diagnosis of Mycobacteria. Clinical Chemistry. 2001;47(5):809-14.
100. Keenan C, Saunders C, Price S, Hinchliffe S, McDonald RA. From Conflict to Bridges: Towards Constructive Use of Conflict Frames in the Control of Bovine Tuberculosis. Sociologia Ruralis. 2020;60(2):482-504.
101. Chandran A, Williams K, Mendum T, Stewart G, Clark S, Zadi S, et al. Development of a diagnostic compatible BCG vaccine against Bovine tuberculosis. Scientific Reports. 2019;9(1):17791.
102. Gormley E, Doyle MB, Fitzsimons T, McGill K, Collins JD. Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam®) assay. Veterinary Microbiology. 2006;112(2):171-9.
103. Goodchild AV, Downs SH, Upton P, Wood JLN, de la Rua-Domenech R. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Veterinary Record. 2015;177(10):258-.
104. Alvarez AH, Estrada-Chávez C, Flores-Valdez MA. Molecular findings and approaches spotlighting *Mycobacterium bovis* persistence in cattle. Veterinary Research. 2009;40(3):22.
105. de la Rua-Domenech R. Human *Mycobacterium bovis* infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. Tuberculosis. 2006;86(2):77-109.
106. Kelley HV, Waibel SM, Sidiki S, Tomatis-Souverbielle C, Scordo JM, Hunt WG, et al. Accuracy of Two Point-of-Care Tests for Rapid Diagnosis of Bovine Tuberculosis at Animal Level using Non-Invasive Specimens. Scientific Reports. 2020;10(1):5441.
107. Amos W, Brooks-Pollock E, Blackwell R, Driscoll E, Nelson-Flower M, Conlan AJK. Genetic Predisposition to Pass the Standard SICCT Test for Bovine Tuberculosis in British Cattle. PLOS ONE. 2013;8(3):e58245.
108. Furniss RCD, Kostrzewa M, Mavridou DAI, Larrouy-Maumus G. The clue is in the lipid A: Rapid detection of colistin resistance. PLOS Pathogens. 2020;16(4):e1008331.