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Rapid Identification of Bacteria by Intact Cell Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry

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Introduction

Characterisation of bacteria using intact cell matrix assisted laser desorption ionisation time of flight mass spectrometry (ICMS) is a newly described rapid identification method. ICMS uses the surface ions generated from the soft-ionisation of the intact bacterial cell to generate apparent reproducible fingerprint spectra within a few minutes ⁽¹⁾. There is sufficient data within the spectrum to identify the bacterial species and further studies have shown that for some bacterial species, the generated fingerprints are sufficiently discriminatory to allow simultaneous sub-typing ^(2,3). To be successful, the bacterial mass spectral fingerprint must be reliably matched to a spectrum of the same species in a database. A large database of quality controlled spectral fingerprints of bacteria of known provenance (NCTC strains) was created and challenged with a data from a selection of the same bacterial strains generated at a different point in time, on a different instrument. In addition, the database was challenged with several clinical isolates. This study describes the preliminary findings.

Method

All bacterial strains used to populate the database were obtained from the National Collection of Type Cultures (NCTC), London and analysed at Manchester Metropolitan University and NCTC, London. They were reconstituted using 0.5ml nutrient broth and sub-cultured three times on Columbia Blood Agar (Oxoid) prior to analysis. They were incubated at 37°C (or appropriate) for 24hrs (or appropriate) in air (or appropriate).

Preparation of the bacteria for analysis by MALDI-TOF MS

Single colonies of bacteria were removed from the plates with a sterile loop and applied directly on to the sample targets. Twelve target wells were used for each organism to be entered into the database. A 1µl aliquot of either a-cyano-4-hydroxycinnamic acid (aCHCA) (Sigma), for Gram-negative bacteria, or 5-Chloro-2-mercaptobenzothiazole (Aldrich) for Gram-positive bacteria was applied to each target spot and allowed to dry prior to mass spectrometric analysis. The UV-absorbing matrix solutions used (aCHCA and CMBT) were freshly prepared as saturated solutions in water, methanol, and acetonitrile, 1:1:1 containing 0.1% formic acid and 0.01M 18-crown-6 ether.

Instrument operation

The MALDI TOF-MS analysis was performed using a M@LDI-Linear time-of-flight mass spectrometer (Micromass UK, Ltd., Manchester, UK). A nitrogen laser giving a 337nm output of 3ns pulse width was used. The laser fluence was set just above the threshold for ion production. The mass spectrometer was used in the positive ion detection mode using an acceleration voltage of +15kV. On loading each target plate, automatic, accurate indexing of the sample/reference wells was performed, followed by calibration of the m/z range of the instrument, using the average molecular weights from a standard peptide mixture (bradyknin, angiotensin I, Glu-fibrinopeptide B, renin substrate tetraddecapeptide, ACTH (18-39 clip) all at 1pmol/µl, bovine insulin, 2pmol/µl and ubiquitin, 10pmol/µl). The acquisition mass range was from m/z 500 to 10000Da. For maximum throughput of samples the bacterial mass fingerprints were acquired automatically . Spectra from the reference wells, for lock mass calibration, were also

MicrobeLynx Search Engine and Database

The database was created at MMU using 335 diverse bacterial strains. The quality of the replicates was determined by comparing each of the twelve replicate spectra to each other using the root mean square (RMS) value. An RMS rejection value of 3 was used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. Spectra from 149 of the same NCTC strains used to populate the database were acquired at NCTC, London and used to challenge the performance of the pattern-matching algorithm developed for this purpose. The Genus and species of the closest matches were displayed in the result's browser. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format. In addition, 96 clinical isolates of *Staphylococcus aureus* was used to challenge the database and assess the performance of the software and technique.

Method

Reproducibility

Examples of reproducibility of replicates produced by *E.coli* are shown in Figure 1. Visually the replicates looked very consistent with good quality spectra. QC of the replicates using the RMS values is demonstrated in Figure 2. The green ticks indicate those replicates that are accepted and the red crosses indicate those that have been rejected.





Database generation and challenge with characterised strains

In total 335 strains were added to the database. **Table 1** shows a list of the Genera and number of species entered. Examples of spectra produced from ten different Genera are shown in **Figure 3**. The spectra from 76 Gram-negative and 73 Gram- positive strains produced at NCTC, London were identified using the pattern recognition software and the accuracy of the technique evaluated. Spectra from 50 (33%) bacterial strains matched exactly to the strain in the database with 38 (76%) having a matching probability of > 90%. An additional 25 strains matched to Genus level. There were 74 strains that were mis-identified but on further scrutiny of the data, errors in data production were determined in many instances. An example of a challenge using *E.coli* is shown in **Figure 4**.

Genera	Entries	Genera	Entries	Genera	Entries
Actinobacillus	1	Escherichia	4	Pasteurella	4
Actinomadura	1	Fluoribacter	3	Pediococcus	2
a-cyano & Media		Gordonia	1	Photobacterium	2
Aeromonas	3	Haemophilus	4	Propionibacterium	1
Alcaligenes	1	Klebseilla	4	Proteus	3
Arcanobacterium	1	Kocuria	3	Providencia	3
Bacillus	15	Kurthia	2	Pseudomonas	3
Bacteroides	1	Kytococcus	1	Pyschrobacter	1
Brevibacillus	1	Lactobacillus	6	Rhodococcus	3
Brochothrix	1	Lactococcus	1	Rothia	1
Burkholderia	1	Legionella	7	Salmonella	5
Campylobacter	10	Listeria	17	Serratia	4
Cardiobacterium	1	Listonella	2	Shigella	2
Cedecea	3	Micrococcus	1	Sphingomonas	1
Chromobacterium	1	Moraxella	5	Staphylococcus	51
Citrobacter	4	Morganella	1	Stenotrophomonas	1
CMBT & Media		Mycobacterium	8	Streptococcus	48
Corynebacterium	9	Neisseria	9	Tatumella	1
Eikenella	1	Nocardia	2	Vibrio	12
Enterobacter	3	Oligella	1	Virgibacillus	1
Enterococcus	6	Paenibacillus	3	Yersinia	4
Erysipelothrix	1	Pantoea	1		







Identification of clinical isolates

Of the 96 clinical isolates of *S. aureus* used to challenge the database, 85/96 (88%) were identified cor-rectly with 70% showing a match of >90% probability. Of the 11 strains that were incorrectly identified, 6 were identified correctly as the Genus staphylococci but incorrectly identified the species. An example of a typical challenge browser displaying a result for the *S. aureus* is shown in **Figure 5**.

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Campylobacter species

The Public Health Laboratory, Preston, provided two clinical isolates together with two NCTC strains of Campylobacter jejuni, (NCTC 11168 and 11392) in order to challenge the database. The NCTC strains were not in the database. However when the NCTC strain 11392 was analysed and searched, the best match with a 100% probability was to a *Campylobacter jejuni* subspecies *jejuni* (NCTC 11351). One of the clinical isolates provided also matched the NCTC 11351 strain in the database with a 99% probability of the NCTC 1

ity. The result's browsers are shown in **Figure 6 and 7**. An extended database containing these *Campylobacter* species was created and the data re-searched against this database. The extension of the database has provided further matches against the Campylobacter entries including the Campylobacter jejuni subspecies jejuni NCTC 11351 strain from the release database



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Conclusion

This technique produces a fingerprint of surface moieties desorbed from an intact bacterial cell during the ionisation process within the mass spectrometer. The structure of the bacterial cell envelope is well described and conservation of certain bio-molecules across the species has been shown. There is also known differences across species e.g. lipopolysaccharide, outer membrane proteins, etc that should differentiate between species and perhaps between strains within a single species. The technique has been shown to be very reproducible and because it is so technically easy, cheap and very rapid it could replace many of the conventional laborious, expensive methods used to identify bacteria. The instrumentation and software are designed such that most of the acquisition and processing of the data is automatic. The reproducibility of the spectra produced can be accurately determined using the RMS value allowing the operator to quality control the data prior to entry into the database

This initial study was undertaken to evaluate the technique as a possible identification tool. The preliminary findings were very encouraging with the first challenge identifying 33% of the spectra accurately to the exact strain with a probability of over 90%. However, there were a number of mis-matches and on interrogation of the data used to create the database and data used to challenge the database, discrepancies were found. Some data was poor and some used to challenge the database contained peptide mix used as the calibrant contaminating the wells. On removal of this poor data the accuracy of the technique increased accordingly. The clinical strains of S. aureus used to challenge the database produced very encouraging results with 89% of the strains accurately identified. This was also the case with the small study on Camplyobacter sp where the NCTC strains and a clinical isolate was successfully identified using the technique

The database continues to expand, with all new data cross-referenced for accuracy. It is envisaged that with a larger complement of reference spectra that accuracy of the identification of the diverse numbers of bacteria that exist in the environment should improve.

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