MATRIX ASSISTED LASER DESORPTION/IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY PROVIDES FAST AND RELIABLE IDENTIFICATION OF *STAPHYLOCOCCUS HAEMOLYTICUS*

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ABSTRACT

Staphylococcus haemolyticus **is an emerging pathogen more resistant to antibiotics than most MRSA. It causes symptoms indistinguishable from *Staphylococcus aureus* infections and is responsible for some outbreaks in orthopaedic and neonatal wards mimicking MRSA infection. Analysis of these isolates can mistakenly be perceived and identified as low-grade pathogenic coagulase negative staphylococci. However, a fast and reliable identification is needed in order to institute appropriate antibiotic therapy. To achieve this we have successfully used the "Peterborough Collection" of clinical isolates to establish a MALDI-TOF MS database for *Staphylococcus haemolyticus*.**

Intact *Staphylococcus haemolyticus* cells are transferred from a culture plate to a MALDI target plate and overlaid with the MALDI matrix 5-Chloro-2-mercaptobenzothiazole. The co-crystallised sample is then irradiated with a N_2 laser and the resulting plume of positive ions separated using time-of-flight mass spectrometry. This produces a characteristic mass spectral fingerprint pattern, which forms the basis of identification against a database containing representative spectra of the species. The MALDI-TOF MS technique also allows for high sample throughput and rapid identification of isolates, since the collection and analysis of spectral data against a substantial database requires ~1.5 hours for a 96 well MALDI target plate.

In this study 71 (93.4%) of the 76 clinical strains of *Staphylococcus haemolyticus* were identified correctly against a database of more than 3600 spectral entries representing over 500 different bacterial species, Figure 1. Furthermore conclusive identification was confirmed for the majority of these strains since all 8 top matches were correct to species. Comparison of three misidentified test spectra with their corresponding spectral matches demonstrated unique high mass ions exclusive to the matching of *S. haemolyticus* spectra. Therefore filtering the matches for these ions it is possible to correctly identify 74 (97.4%) of the 76 clinical strains to species level.

MALDI-TOF MS has successfully identified 97.4% clinical isolates of *S. haemolyticus*, demonstrating the technique provides fast reliable identification of this emerging pathogen.

INTRODUCTION

Most coagulase negative staphylococci (CNS) were regarded as non-pathogenic. Recently they have become more important, emerging as nosocomial pathogens for prosthesis and surgical site infections. *Staphylococcus haemolyticus* is the second most commonly isolated CNS and now accounts for about 10% of clinical isolates. It is implicated in infections in immuno-competent patients, immuno-compromised patients and in infections associated with invasive devices and implants[1,2]. Outbreaks have been reported from neonatal intensive care units, surgical intensive care units and oncology units[3,4].

Staphylococcus haemolyticus may mistakenly be identified as MRSA as it has a similar colony morphology and susceptibility pattern. Indeed, it is often more resistant to antibiotics than MRSA; therefore proper identification and susceptibility testing are needed in order to facilitate appropriate choice of antibiotics[5,6].

The objective of this study is to establish a MALDI-TOF MS fingerprint database for identification of *Staphylococcus haemolyticus*. The MALDI-TOF MS technique offers fast, reliable and inexpensive identification of this emerging pathogen, suitable for high sample throughput and epidemiological investigation.

METHODS

Culture conditions:

1. NCTC strains

- Reconstitution of ampoules of NCTC strains was carried out in accordance with NCTC guidelines, 'Opening of Ampoules'. (www.hpa.org.uk)
- Followed generally by an incubation of 24 hours at 37 °C on Columbia Blood agar (CBA) containing 5% (v/v) horse blood, in an aerobic atmosphere. (CBA: Oxoid, Basingstoke, UK.)
- Two further sub-cultures were carried out prior to MALDI-TOF-MS analysis by either Manchester Metropolitan University (MMU) Manchester, UK or Health Protection Agency (HPA) London, UK.

2. "Peterborough Collection" of Clinical Isolates

- The "Peterborough Collection" of 100 clinical isolates of *Staphylococcus haemolyticus* were either reconstituted from freeze dried ampoules as above or subcultured from Nutrient Agar slopes.
- Each isolate was inoculated onto two Columbia Blood agar (CBA) plates containing 5% (v/v) horse blood (CBA: Oxoid, Basingstoke, UK.), and incubated simultaneously for 24 hours at 37 °C; the first plate in an aerobic atmosphere; the second in an aerobic atmosphere enriched with 5% Carbon dioxide.
- Two further sub-cultures were carried out prior to MALDI-TOF MS analysis
- All clinical isolates were characterized and identified as *Staphylococcus haemolyticus* using the Staph API system (BioMerieux, France).

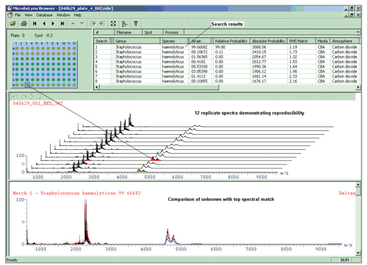

Figure 1: Browser results demonstrating the top 8 closest matches as Staphylococcus haemolyticus; the reproducibility of the 12 replicate spectra; and the similarity of the database and unknown test spectra.

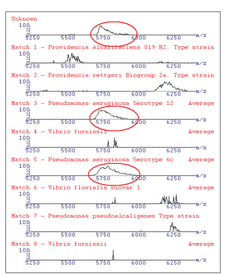

Figure 2: Spectral matches demonstrating the appropriate filtering of the top results for Pseudomonas aeruginosa NCTC 10332 for the distinctive mass peak at ~5,750 Da.

3. Test strains

- Seventy-six clinical strains of *Staphylococcus haemolyticus* isolates were used as test strains
- Two NCTC strains *Micrococcus lylae* NCTC 11037 and *Pseudomonas aeruginosa* NCTC 10332 were used as control test strains.
- The NCTC strains were cultured as above initially from freeze-dried ampoules and subsequently from frozen beads.
- The test strains were cultured as for the "Peterborough Collection" initially from freeze-dried ampoules and subsequently from nutrient agar slopes.

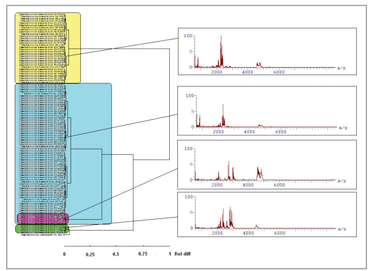

Figure 3: Cluster analysis and representative spectral fingerprints for Staphylococcus haemolyticus isolates cultured from enriched carbon dioxide atmosphere.

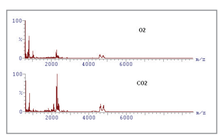

Figure 4: Comparison of spectral fingerprint for Staphylococcus haemolyticus 01.53568 cultured aerobically (O_2) and in 5% enriched carbon dioxide atmosphere (CO_2), demonstrating the increase in peak intensity.

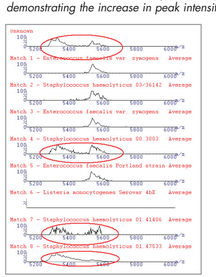

Figure 5: Spectral matches demonstrating the appropriate filtering of the top results for the distinctive mass peaks in the region of 4,500 – 5,000 Da.

Preparation of bacterial samples for MALDI-TOF MS analysis

- Using a 1µL culture loop, bacterial colonies from the culture plate were inoculated onto 12 wells of a MALDI target plate. (i.e. Twelve wells per bacterial sample)
- Samples were air-dried for at least 1 hour.
- Then overlaid with 1µL aliquot of a saturated matrix solution; 5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich Chemical Company) for Gram-positive bacteria; α-cyano-4-hydroxycinnamic acid; Sigma-Aldrich Chemical Company) for Gram-negative bacteria
- Matrix solvent acetonitrile: methanol: water (1:1:1) with 0.1% (v/v) formic acid and 0.01M 18-crown-6-ether.

MALDI-TOF MS analysis

- Analysis was performed using a M@LDI-Linear time of flight mass spectrometer (Waters Corporation, Manchester, UK).
- A nitrogen laser giving a 337nm output of 3ns pulse width.
- Laser fluence was set to just above the threshold for ion production in the positive ion detection mode.
- Acceleration voltage of 15kV
- On loading each target plate, automatic, accurate indexing of the sample/reference wells was performed.
- Mass calibration was performed using the average molecular weights from a (1:1) standard peptide mixture (bradykinin, angiotensin I, gul-fibrinopeptide B, rennin substrate tetra decapeptide, ATCH (18-39 clip) all at 1pmol/mL, bovine insulin 2pmol/mL and ubiquitin 10pmol/mL) : matrix, saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile: methanol: water (1:1:1) with 0.1% (v/v) formic acid and 0.01M 18-crown-6.
- Data acquisition mass range was from m/z 500 to 10,000 Da.
- Bacterial mass fingerprints, and spectra from reference wells, for lock mass calibration, were automatically acquired using the MAXspec real-time data selection algorithm to optimise the bacterial fingerprint in the mass range 800-3,000 Da.

Database construction

- A database was constructed which included 3,424 spectral entries from NCTC strains and 202 spectral entries from clinical isolates of the "*Staphylococcus haemolyticus* Peterborough Collection".
- Replicates of twelve spectra, per bacterial strain, were compared for reproducibility using the root mean square (RMS) value; this value is obtained by comparing each replicate in turn with the average of the other 11 replicates.
- An RMS rejection value of three was used to identify outliers.
- Outliers were excluded from addition to the database.
- The remaining replicates were then combined to give a representative average spectrum, which was added to the database.

Data analysis using the Waters MicrobeLynx™ software.

- The average spectrum of each test strain was searched against the database of 3,624 spectral entries.
- The search uses a pattern recognition algorithm to compare the mass and intensity data in the test spectrum with each database entry.
- The top 8 database matches are tabulated in order of probability.
- A display of the tabulated results, the test spectrum and database spectrum are presented in a browser format, Figure 1.

RESULTS

1. The study was carried out over a period of ~10 months, consequently to ensure reproducibility of MALDI-TOF MS spectra, each set of samples sent for analysis included two NCTC strains as control. The spectral fingerprints for these strains remained consistent throughout the study, **with both *Micrococcus lylae* NCTC 11037 and *Pseudomonas aeruginosa* NCTC 11032 achieving 100% successful identification against the database.** However, three results for and Pseudomonas aeruginosa NCTC 11032 required filtering re high mass peaks, Figure 2, and 1 result was discarded due to contamination.
2. In general **the *Staphylococcus haemolyticus* isolates produced similar and distinctive spectral fingerprints** as demonstrated by isolates from the cluster analysis of the carbon dioxide enriched cultures, Figure 3.
3. The isolates cultured in the **enriched carbon dioxide atmosphere generally produced more intense spectral peaks** than the corresponding aerobic cultures, Figure 4.
4. **Correct first match** was achieved for 71 **(93.4%)** of the 76 clinical test strains of *Staphylococcus haemolyticus* against a database of **more than 3,600 spectral entries representing over 500 different bacterial species.**
5. **Filtering the top matches** with respect to unique high mass ions resulted **in 3 further correctly identified test strain** increasing the **total** number of correctly identified to 74 **(97.4%)**, Figure 5.
6. Majority of **database matches were conclusive** with top 8 matches to *Staphylococcus haemolyticus*, Figure 1.
7. Majority of **database matches** were to spectral fingerprints cultured in a **similar atmosphere** (e.g. O_2 test strains matched O_2 database entries).

CONCLUSION

- **The technique provides fast and reliable species identification.**
- **MALDI-TOF MS has successfully identified 97.4% of clinical *S. haemolyticus* isolates**
- **Identification of Clinical Isolates is possible using MALDI-TOF MS.**

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