

## ABSTRACT

## Introduction

*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 may mistakenly be perceived and identified as low-grade pathogenic coagulase negative *staphylococci*. 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*.

## Methods

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 (CMBT). The co-crystallised sample is then irradiated with a N<sub>2</sub> 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.

## Preliminary results

In this study 85 (94.4%) of the 90 clinical strains of *Staphylococcus haemolyticus* were identified correctly against a database of >3600 spectral entries representing over 500 different bacterial species. 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 *S. haemolyticus* spectra. Therefore filtering the matches for these ions it is possible to correctly identify 88 (97.8%) of the 90 clinical strains.

## 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<sup>1,2</sup>. Outbreaks have been reported from neonatal intensive care units, surgical intensive care units and oncology units<sup>3,4</sup>.

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

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](http://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.

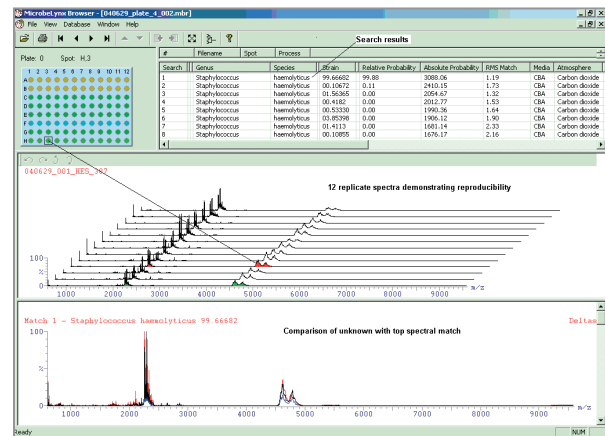


Figure 1. Browser results demonstrating the top 8 closest matches as *Staphylococcus haemolyticus*; the reproducibility of the 12 replicate spectra; and the similarity of the database and unknown test spectra.

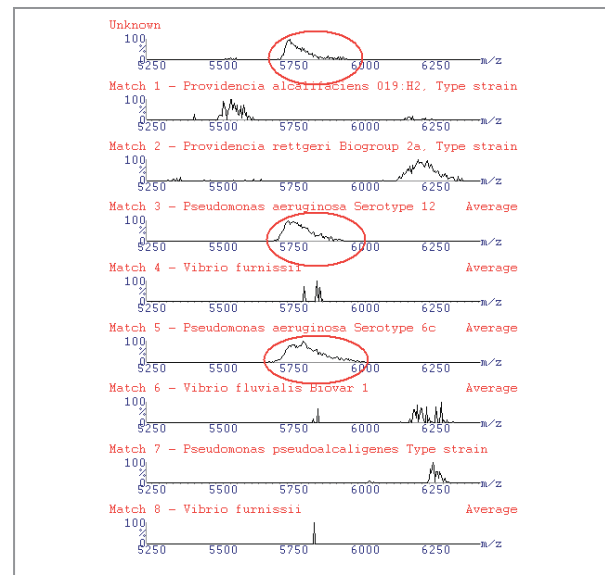


Figure 2. Spectral matches demonstrating the appropriate filtering of the top results for *Pseudomonas aeruginosa* NCTC 10332 for the distinctive mass peak at ~5,750 Da.

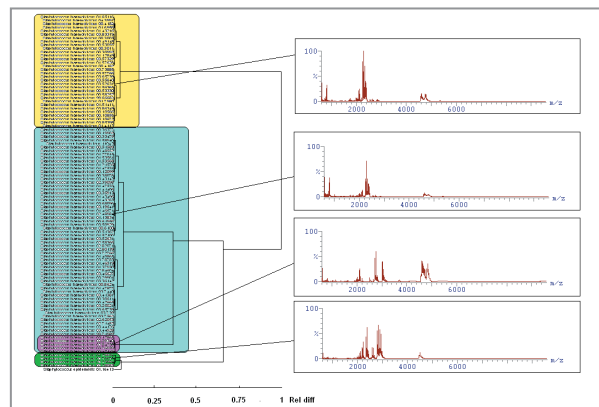


Figure 3. Cluster analysis and representative spectral fingerprints for *Staphylococcus haemolyticus* isolates cultured from enriched carbon dioxide atmosphere.

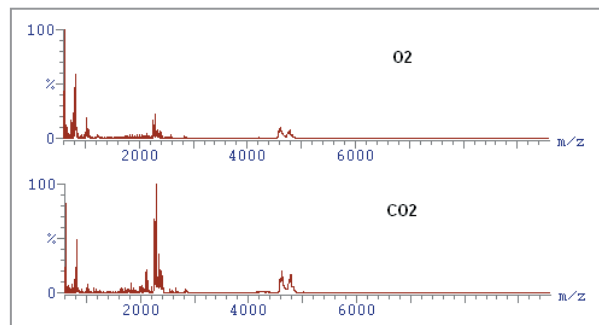


Figure 4. Comparison of spectral fingerprint for *Staphylococcus haemolyticus* 01.53568 cultured aerobically (O<sub>2</sub>) and in 5% enriched carbon dioxide atmosphere (CO<sub>2</sub>), demonstrating the increase in peak intensity.

- 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).

## 3. Test strains

- Ninety 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.

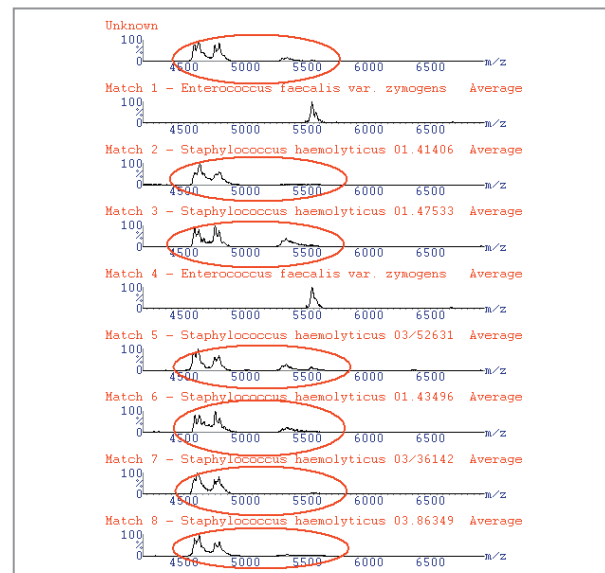


Figure 5. Spectral matches demonstrating the appropriate filtering of the top results for the distinctive mass peaks in the region of 4,500–5,000 Da.

- The test strains were cultured as for the "Peterborough Collection" initially from freeze-dried ampoules and subsequently from nutrient agar slopes.

## Preparation of bacterial samples for MALDI-TOF-MS analysis

- Using a 1 µL 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 1 µL 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 0.01 M 18-crown-6.

## 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 337 nm output of 3 ns pulse width.
- Laser fluence was set to just above the threshold for ion production in the positive ion detection mode.
- Acceleration voltage of 15 kV.

- 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 1 pmol/µL, bovine insulin 2 pmol/µL and ubiquitin 10 pmol/µL) : matrix, saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile: methanol: water (1:1:1) with 0.1% (v/v) formic acid and 0.01 M 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 MicroBelyn<sup>TM</sup> 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 data-base spectrum are presented in a browser format, Figure 1.

## RESULTS

- 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 or 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.

- 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.
- The isolates cultured in the enriched carbon dioxide atmosphere generally produced more intense spectral peaks than the corresponding aerobic cultures, Figure 4.
- Correct first match was achieved for 85 (94.4%) of the 90 clinical test strains of *Staphylococcus haemolyticus* against a database of more than 3,600 spectral entries representing over 500 different bacterial species.
- 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 88 (97.8%), Figure 5.
- Majority of database matches were conclusive with top 8 matches to *Staphylococcus haemolyticus*, Figure 1.
- Majority of database matches were to spectral fingerprints cultured in a similar atmosphere (e.g. O<sub>2</sub> test strains matched O<sub>2</sub> database entries).

## CONCLUSION

- The technique provides fast and reliable species identification.
- MALDI-TOF MS has successfully identified 97.8% of clinical *S. haemolyticus* isolates.
- Identification of Clinical Isolates is possible using MALDI-TOF MS.

## References

- Isaac D.W., Pearson T.A., Hurwitz C.A., Patrick C.C., Clinical and microbiologic aspects of *Staphylococcus haemolyticus* infections. *Pediatr Infect Dis J*, 1993 Dec;12(12):1018-21.
- Degener J.E., Heck M.E., van Leeuwen W.J., Heemskeek C., Crielaard A., Joosten P., Caesar P., Nosocomial infection by *Staphylococcus haemolyticus* and typing methods for epidemiological study. *J Clin Microbiol*. 1994 Sep;32(9):2260-5.
- Perl T.M., Kruger W.A., Houston A., Boyken L.D., Pfaller M.A., Herwaldt L.A., Investigation of suspected nosocomial clusters of *Staphylococcus haemolyticus* infections. *Infect Control Hosp Epidemiol*. 1999 Feb;20(2):128-31.
- Sismey A.W., Latham S., Nielsen P.B., Clark G., Outbreaks of *Staphylococcus haemolyticus* mimicking MRSA. Public Health Laboratory Service 26th Annual Scientific Conference, University of Warwick 2001.
- Froggatt J.W., Johnston J.L., Galetto D.W., Archer G.L., Antimicrobial Resistance in Nosocomial Isolates of *Staphylococcus haemolyticus*. *Antimicrobial Agents and Chemotherapy* Apr.1989; 460-466.
- Sismey A.W., Nielsen P.B., The susceptibility of *Staphylococcus haemolyticus* to 13 antibiotics. ICAAC 2002, San Diego, California, US.

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