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APPLICATION OF A MALDI-TOF MASS SPECTRAL DATABASE FOR THE RAPID IDENTIFICATION OF MICROBES: A DEGREE OF CERTAINTY

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INTRODUCTION

A mass spectral database of over 2000 clinical, environmental and foodborne pathogens has been developed using matrix assisted linear desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The organisms represented in the database are currently all from the National Collection of Type Cultures, and represent a world-renowned well-characterized collection. Laser interrogation of intact microbial cells, taken directly from the culture plate onto the MALDI target plate and co-crystallized within a MALDI matrix solution of either α-cyano-4-hydroxycinnamic acid (αCHCA) for Gram-negative bacteria, or 5-Chloro-2-mercaptobenzothiazole for Gram-positive bacteria, causes ionization of the surface molecules characteristic of the organism[1]. The mass spectral fingerprint pattern produced is automatically collected to provide quality spectra using the dedicated MicrobeLynx™ software. These spectra are further examined for reproducibility before database addition[2]. The same process can be used to collect spectra for unknown isolates and the software enables these to be identified against the library of 2000 microbes in seconds, making this one of the most rapid ID techniques[3]. The technique also has the advantage of minimum sample preparation. For reliable ID, the number of microbial species represented in the database needs to be sufficient to cover the wide diversity contained within each species whilst still maintaining the unique pattern of biomarkers. If this is the case then identification of unknown samples can be established with a high degree of certainty, with the top five spectral matches suggesting the same species[4]. This study demonstrates the increased confidence associated with identification as the number of strains per species is increased.

METHOD

Bacterial Culture Conditions

1.Database

All the NCTC strains for the database were prepared from freeze-dried ampoules as follows:

- Reconstitution of ampoules in accordance with NCTC guidelines, 'Opening of Ampoules'[5].
- Incubation generally 24 hours at 37°C on Columbia Blood agar (CBA, Oxoid, Basingstoke, UK) containing 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks. UK.), as supplied by the Public Health Laboratory Service accredited laboratories (Chester, UK), in an aerobic atmosphere.*
- Two further sub-cultures prior to MALDI-TOF MS analysis.
- A significant number of database entries were also incubated for 16 hours at 35°C on Cysteine Lactose Electrolyte Deficient agar (CLED, Oxoid, Basingstoke, UK) in an aerobic atmosphere. Some of these entries (e.g. *Proteus mirabilis*) were duplicated using CLED agar containing Andrades indicator. Some organisms required anaerobic conditions and were therefore cultured accordingly.

2.Test NCTC strains and Clinical isolates

Prepared as above either from ampoules or from nutrient agar slopes[6] and analyzed against the 3 database releases to find the top 8 best matches.

Preparation for MALDI-TOF MS analysis of NCTC strains and Clinical isolates

- Using a 1µL culture loop, several bacterial colonies were applied to 12 target plate wells. (Twelve wells per strain)
- Samples were air-dried for at least 1 hour.

- Overlaid with 1µL aliquot of matrix solvent* containing either 5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich Chemical Company) for Gram-positive bacteria or α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemical Company) for Gram-negative.
- Allowed to air dry.

(*Acetonitrile: Methanol: Water (1:1:1) with 0.1% (v/v) formic acid and 0.01M 18-crown-6.)

INSTRUMENTATION

Analysis was performed using a Waters® Micromass® M@LDI-Linear time of flight mass spectrometer using:

- A nitrogen laser giving a 337 nm output of 3 ns pulse width.
- Laser fluence just above the threshold for ion production in the positive ion detection mode.
- An acceleration voltage of +15 kV.
- Automatic, accurate indexing of the sample/reference wells.
- Mass calibration using the average molecular weights from a (1:1) standard peptide mixture; (bradykinin, angiotensin I, glu-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, α-cyano-4-hydroxycinnamic acid.
- A data acquisition mass range from m/z 500 to 10,000 Da.
- Automatic collection of bacterial mass fingerprints, and spectra from reference wells for lockmass calibration, using the MAXspec™ real-time data selection algorithm to optimize the bacterial fingerprint in the mass range 800-3000 Da.

DATABASE CONSTRUCTION

- Three database were constructed;
  - Database release 2001 (307 spectral entries).
  - Database release 2002 (1099 spectral entries).
  - Database release 2003 (2159 spectral entries).
- Each database entry consisted of a representative average spectrum derived from combining up to 12 replicate spectra for each bacterial strain. Significant outliers were eliminated using a root mean square (RMS) rejection value of 3, Figure 1.

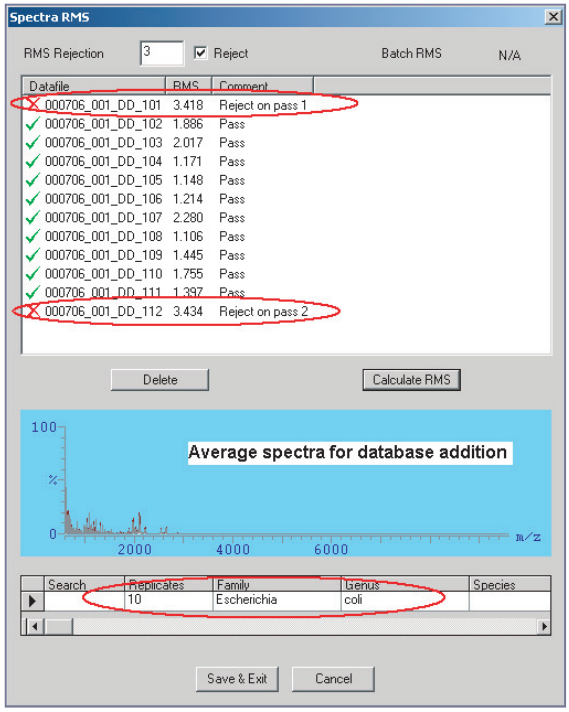
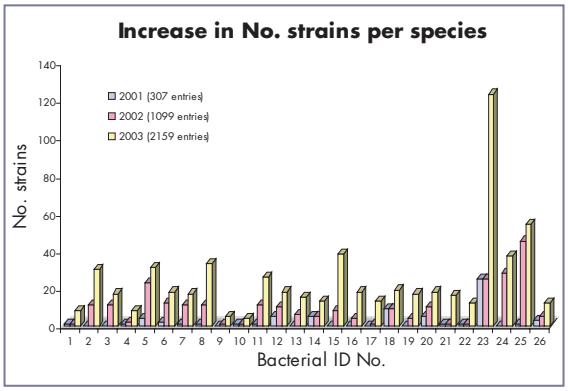
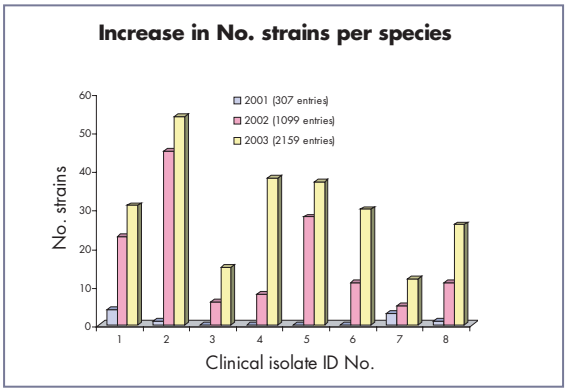


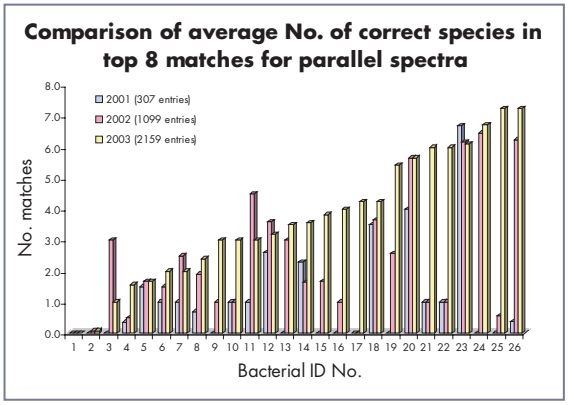
Figure 1. RMS values of each replicate of E.coli NCTC 9001 compared to the average spectra (2 spectra rejected) in the browser.



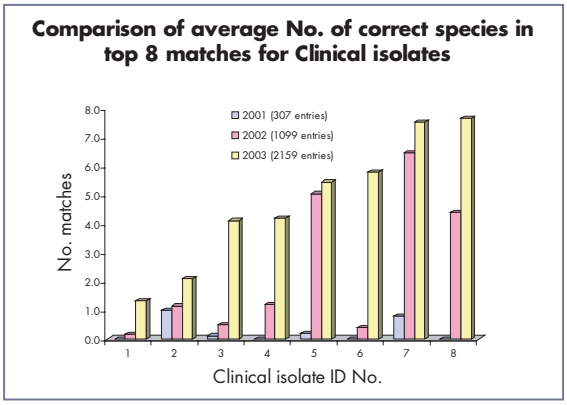
Graph 1. Increase in the No. of strains per species relating to the parallel data for databases 2001, 2002 & 2003.



Graph 3. Increase in the No. of strains per species relating to the clinical data for databases 2001, 2002, 2003.



Graph 2. Improved No. of correct species matches as the number of database entries is increased.



Graph 4. Improved No. of correct species matches as the number of database entries has increased.

ID	Genus	Species	No. strains tested
1	*Corynebacterium*	*xerosis*	1
2	*Enterobacter*	*cloacae*	12
3	*Providencia*	*stuartii*	1
4	*Bacillus*	*pumilus*	14
5	*Klebseilla*	*pneumoniae*	6
6	*Campylobacter*	*jejuni*	2
7	*Providencia*	*stuartii*	2
8	*Pseudomonas*	*aeruginosa*	10
9	*Corynebacterium*	*renale*	1
10	*Corynebacterium*	*jeikeium*	1
11	*Proteus*	*mirabilis**	2
12	*Pasteurella*	*multocida*	6
13	*Klebseilla*	*pneumo niae*	2
14	*Bacillus*	*cereus*	14
15	*Enterobacter*	*cloacae*	6
16	*Yersinia*	*pseudotuberculosis*	1
17	*Acinetobacter*	*calcoaceticus*	3
18	*Listeria*	*monocytogenes*	12
19	*Chryseobacterium*	*meningosepticum*	7
20	*Salmonella*	*choleraesuis*	9
21	*Bacillus*	*licheniformis*	2
22	*Corynebacterium*	*ulcerans*	1
23	*Staphylococcus*	*aureus*	17
24	*Escherichia*	*coli*	15
25	*Escherichia*	*coli*	16
26	*Proteus*	*mirabilis**	8
		Total	172

Key to Bacterial isolate ID No.

ID	Genus	Species	No strains tested
1	*Klebsiella*	*pneumoniae*	12
2	*Escherichia*	*coli*	20
3	*Klebsiella*	*pneumoniae*	16
4	*Enterobacter*	*cloacae*	5
5	*Escherichia*	*coli*	20
6	*Enterobacter*	*cloacae*	5
7	*Proteus*	*mirabilis*	15
8	*Proteus*	*mirabilis*	15
		Total	108

Key to clinical isolate ID No.

Denotes spectra generated from bacteria cultured on CLED agar

* Matches on both CBA, & CLED as spectral pattern very similar for both media types

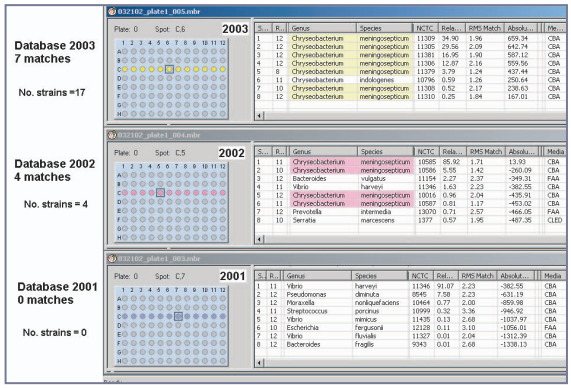


Figure 2. Database search results of parallel data for Chryseobacterium meningosepticum NCTC 10586 demonstrating the increased number of correct species in the top 8 matches as the no. of strains are increased.

RESULTS

The number of strains per bacterial species represented in the initial database of 2001 has increased significantly in the subsequent releases of 2002 and 2003. A selection of these strains have been tested against the 3 database releases using parallel* spectra generated at either the Manchester Metropolitan University, Manchester, or the Health Protection Agency, London. A limited number of clinical isolates have also been tested (Chester PHLS). The increase in the number of strains per species in each database together with the results of the matches for the species tested is presented in Graphs 1 & 2 for the parallel study and Graph 3 & 4 for the clinical isolates.

The results demonstrate:

- As the number of strains per species in the database increases, the number of correct species matches in the top 8 increases, Figure 2.
- The increase in the number of correct species in the top 8 matches demonstrates the increase in the degree of certainty as to the identity of a bacterium, Graph 1 & 2.
- The number of strains per species required for conclusive identification (i.e. ≥ 4/8 correct matches) is varied & dependent upon the species. Nevertheless the parallel data suggests a minimum of 18 is generally required.
- The results for bacterial ID nos. 3 & 11, Graph 2 (*Providencia stuartii* & *Proteus mirabilis* on CLED) are anomalous, the average no. of correct species has not improved from the 2002 to 2003 release, suggesting:

  - The number of strains per species needs to be increased.

  - More strains need to be tested.

- For bacterial ID nos. 7 & 12 (*Providencia staurtii* & *Pasteurella multocida* on CBA) the average no. of correct species for 2002 & 2003 are very similar, suggesting more strains are required for these species.
- Identification of clinical isolates can be achieved with a high degree of certainty if the database contains a significant number of strains per species and the top matches are the same species, Graph 3 & 4.
- Identification of clinical isolate ID nos. 1 & 2 (*Klebsiella pneumonia* & *Escherichia coli* on CBA) were not conclusive. These were identified more conclusively when cultures on CLED agar[7].

*Parallel spectra are defined as spectra produced in a parallel study on a different site, using a different instrument & operator.

CONCLUSION

An increase in the number of strains per species in the database:

- Increases the degree of certainty of identification.
- Allows for rapid identification of clinical isolates with a high degree of certainty.
- A minimum of 18 strains per species is required for the majority of species tested.

ACKNOWLEDGEMENTS

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