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OPTIMIZATION OF A DATABASE FOR RAPID IDENTIFICATION OF INTACT BACTERIAL CELLS OF *ESCHERICHIA COLI* BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Presented at ASMS, Montréal, Canada, 8th-12th June 2003

OVERVIEW

Introduction:

The technique compares the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectral (MS) profile produced from the ionizable surface components of an organism with those contained within a database and identifies a list of matching organisms. Preliminary results of challenging a database of ~1500 microbial spectra cultured on Columbia Blood Agar (CBA), identifies several strains of the same species for many organisms. Identification is therefore conclusive. For biochemically similar microbes e.g. *Escherichia coli* and the *Citrobacter* species, differentiation is more subtle. This paper demonstrates the ability to differentiate biochemically similar species by optimizing the choice of culture media.

The impact of this more specialized database upon the identification of *Escherichia coli* is investigated.

Methods and Instrumentation:

Strains from the National Collection of Type Cultures were propagated using strict protocols appropriate to the CBA or CLED (Cysteine Lactose Electrolyte Deficient agar) database requirements. Microbial samples were innoculated onto the target plate and co-crystallized with either α-cyano-4-hydroxycinnamic acid (αCHCA), for Gram-negative bacteria, or 5-Chloro-2-mercaptobenzothiazole (CMBT) for Gram-positive bacteria, before MALDI-TOF MS analysis using Waters MALDI L™ instrument. The spectra were examined for reproducibility using the MicrobeLynx™ software before addition to either the CBA or CLED database. The software cluster algorithm was used to compare the same strains from each database.

The clinical samples were cultured on both CBA and CLED media. The spectral profiles were simultaneously collected and compared with the database entries to establish the most appropriate match.

Preliminary Data:

Comparison of the cluster analysis for the same strains of *Escherichia coli* and *Citrobacter koseri* cultured using CBA and CLED agar were compared. Both produced two main cluster groups.

The results using CBA demonstrate that 6 of the *Escherichia coli* form a separate group, and 8 *Citrobacter* species together with 3 *Escherichia coli* species form a second, much less distinctive group.

The results using the CLED agar were by contrast very decisive, with 8 of the 9 *Escherichia coli* clustering into one distinct group and all the *Citrobacter koseri* species into another distinct group. Only 1 *Escherichia coli* grouped with the *Citrobacter* species.

The results suggest that differences between *Escherichia coli* & *Citrobacter* species are more pronounced when the bacterium are cultured on CLED agar and is likely to aid identification of clinical isolates for these species. This is confirmed by the analysis of 20 clinical isolates of *Escherichia coli*. Correct identification is achieved in 75% and 35% of the cases for the duplicate CLED and CBA cultures respectively.

INTRODUCTION

Laser interrogation of the surface of intact bacterial cells provides a unique fingerprint pattern of biomarkers, which has the potential to be used as the basis for identification[1-4]. A unique database containing bacterial strains from the National Collection of Type Cultures (NCTC) has been constructed using strict protocols, and has been investigated with respect to the reproducibility of the spectra. The ability to identify bacteria from parallel studies and quality assurance data together with the data for clinical isolates has also been demonstrated[5-10]. For these studies the database tested consisted of over 100 different genera, containing 900 spectral entries. The majority of organisms were cultured under the same conditions

on CBA to make identification of an unknown as simple as possible. Only the Gram stain of the organism is required prior to analysis. MALDI-TOF MS identification of the intact bacterium is performed in a matter of minutes, making this one of the most rapid identification techniques currently available. During the testing of the database several strains of the same species were often identified within the top matches[11]. Identification is therefore conclusive. However discrimination of some closely related species proved more problematic. In particular *Escherichia coli* was incorrectly identified on a number of occasions[8]. One of the most consistent top matches for this species appeared to be *Citrobacter koseri*, Figure 1. This is not surprising given the biochemical similarity between these species[12].

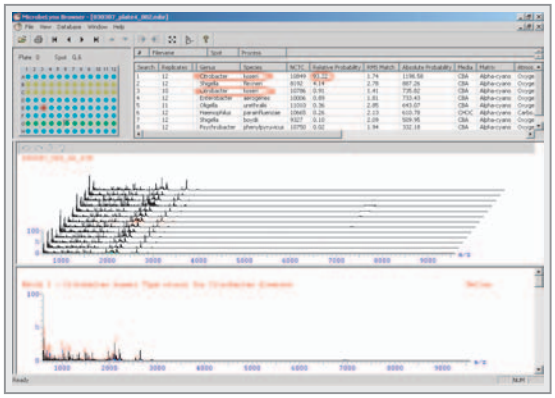

Figure 1. Results demonstrating the top matches for Escherichia coli NCTC 8621 against databases approaching 2000 spectral entries.

This study investigates the potential for improved discrimination between MALDI-TOF mass spectra for these species when more specialized (CLED) culture conditions are used. The choice of conditions reflects those generally used for the identification of clinical organisms from urine samples in which they are commonly found[13].

METHOD

Bacterial Culture Conditions

1. Database 1: CBA

All the NCTC database strains for database 1 were prepared from freeze-dried ampoules as follows:

- Reconstitution of ampoules in accordance with NCTC guidelines, 'Opening of Ampoules'[14].
- 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.

2. Database 2: CLED

The NCTC database strains for database 2 were generally prepared from freeze-dried ampoules, as follows:

- Reconstitution of ampoules in accordance with NCTC guidelines, 'Opening of Ampoules'.[14]
- Incubation 16 hours at 35°C on Cysteine Lactose Electrolyte Deficient agar (CLED, Oxoid, Basingstoke, UK) in an aerobic atmosphere.
- Two further sub-cultures prior to MALDI-TOF MS analysis.

3. Clinical samples

Clinical urine isolates (identified as *Escherichia coli* using the API identification system[15], stored on nutrient agar slopes, were revived by culturing on to Columbia Blood agar & incubating at 37°C for 24 hours in an aerobic atmosphere

- The revived samples were simultaneously sub-cultured onto CBA & CLED agar
  - CBA samples were incubated at 37°C for 24 hours in an aerobic atmosphere.
  - CLED samples were incubated at 35°C for 16 hours in an aerobic atmosphere.

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

- Using a 1 µL 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 1 µL aliquot of matrix solvent* containing either 5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich Chemical Company) for Gram-positive bacteria or a-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 0.01 M 18-crown-6.)

INSTRUMENTATION

Analysis was performed using a Waters® Micromass® MALDI L 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, 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, 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

- Two databases were constructed;
  - Database 1: CBA: 1358 spectral entries covering over 100 different genera.
  - Database 2: CLED: 422 spectral entries, covering ~ 20 different genera.
- Each database entry consisted of a representative average spectrum derived from combining up to 12 replicate spectra obtained for each bacterial strain. Significant outliers were eliminated using a root mean square (RMS) rejection value of 3.

Data analysis

1. Comparison of typical spectra for *Escherichia coli* & *Citrobacter koseri* from database 1: CBA & database 2: CLED.
2. Evaluation of the differentiation between the spectra for the same strains of *Escherichia coli* & *Citrobacter koseri* from database 1 (CBA) and database 2 (CLED) using a simple cluster algorithm based upon the spectral RMS calculation. Each node of the dendogram is singly linked, & clusters of organisms are grouped together using a simple average proximity.
3. Assessment of the search results for the average spectrum of *Escherichia coli* from clinical isolates cultured from CBA & CLED against the combined databases 1 & 2, containing a total of 1770 spectral entries, using a pattern recognition algorithm.
4. Comparison of the search results for expanded database containing 2159 spectral entries.

RESULTS

1. Comparison of the database mass spectral profile for *Escherichia coli* NCTC 9703 & *Citrobacter koseri* NCTC 11073 on both CBA & CLED agar are demonstrated in Figures 2a, 2b, 3a & 3b.
   - Figure 2a confirms the similarity of the two species using CBA cultures, producing poor discrimination.
   - Figure 2b identifies the significant difference between the two species when the culture media is changed to CLED, producing excellent discrimination.

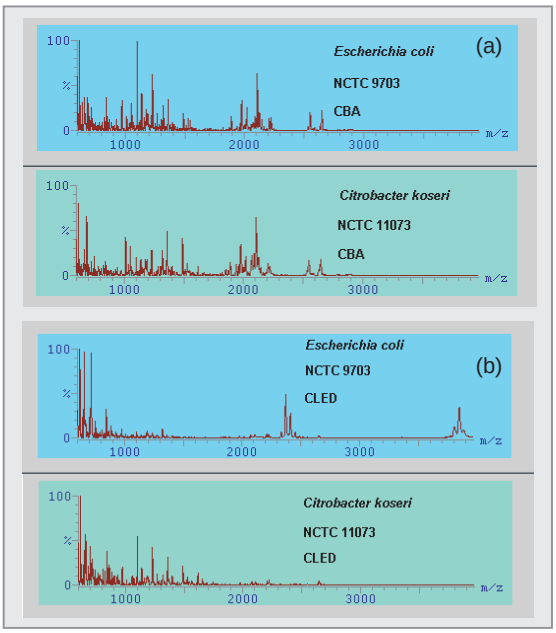

Figure 2. Comparison of typical database mass spectral profiles for Escherichia coli & Citrobacter koseri on both a) CBA & b) CLED agar.

- Figure 3a & 3b verify the respective CBA & CLED spectra are significantly different.

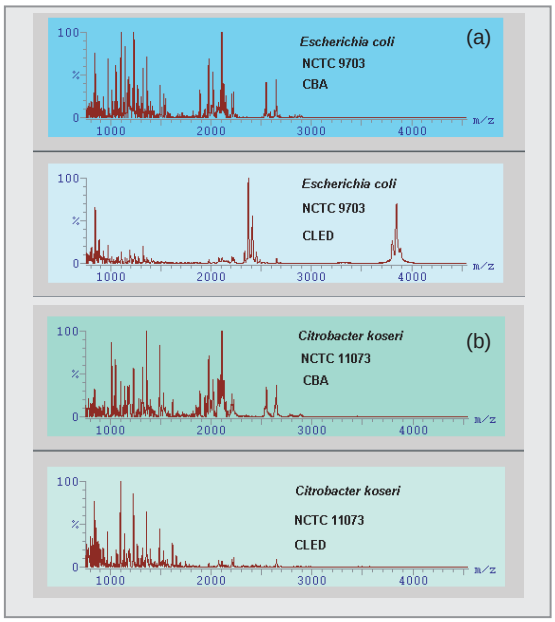

Figure 3. Comparison of typical spectra of a) Escherichia coli & b) Citrobacter koseri on CBA & CLED agar.

2. Figure 4a & 4b are dendograms produced from the cluster analysis of 9 strains of *Escherichia coli* & 8 strains of *Citrobacter koseri* for database 1: CBA & database 2: CLED respectively. Figure 6a & 6b are the respective dendograms produced when the corresponding number of *Escherichia coli* strains is increased to 37.

   - Figure 4a reveals 2 distinctive clusters for database 1: CBA:
     - The first contains 6 *Escherichia coli*.
     - The second contains all 8 *Citrobacter* species together with 3 *Escherichia coli*.
     - Based on relative differences, the second group shows more heterogeneity.

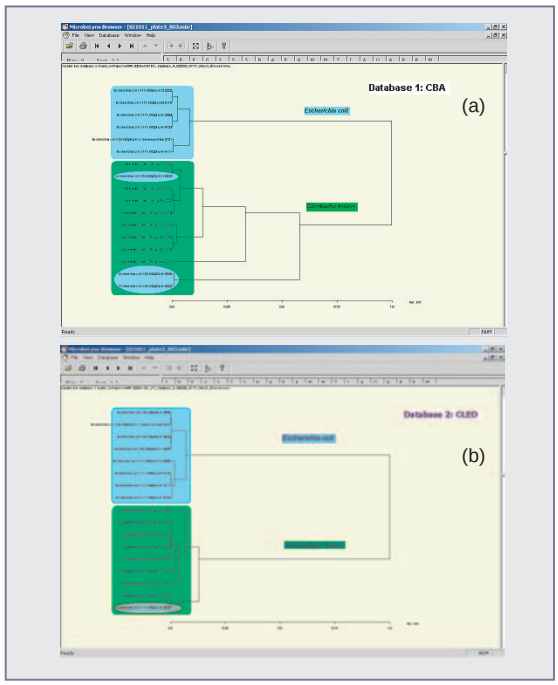

Figure 4. Dendograms produced from the cluster analysis of 9 strains of Escherichia coli & 8 strains of Citrobacter koseri for a) database 1: CBA & b) database 2: CLED respectively.

- Figure 4b also reveals 2 distinctive clusters for database 2: CLED:
  - The first contains 8 *Escherichia coli*
  - The second contains all 8 *Citrobacter* species together with 1 aberrant Escherichia coli
  - Both clusters showed a high degree of homogeneity.
  - The aberrant *Escherichia coli* NCTC 8333 spectrum is anomalous & closely resembles those of *Citrobacter koseri*, Figure 5.

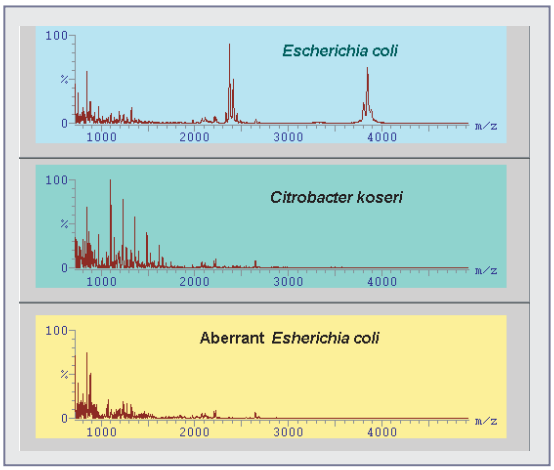

Figure 5. Abberant Escherichia coli NCTC 8333 spectrum from database 2: CLED; demonstrating the lack of distinctive "Escherichia coli" peaks around 2400 & 3850 Da.

- Figure 6a containing 37 *Escherichia coli* & 8 *Citrobacter koseri* species reveals 2 main clusters for database 1: CBA.
  - The first cluster contains 2 sub divisions; 7 *Citrobacter koseri* & 2 *Escherichia coli* species; 8 *Escherichia coli* & 2 *Citrobacter koseri*.
  - The second contains the remaining 27 *Escherichia coli*.
  - Both clusters demonstrate a degree of heterogeneity.

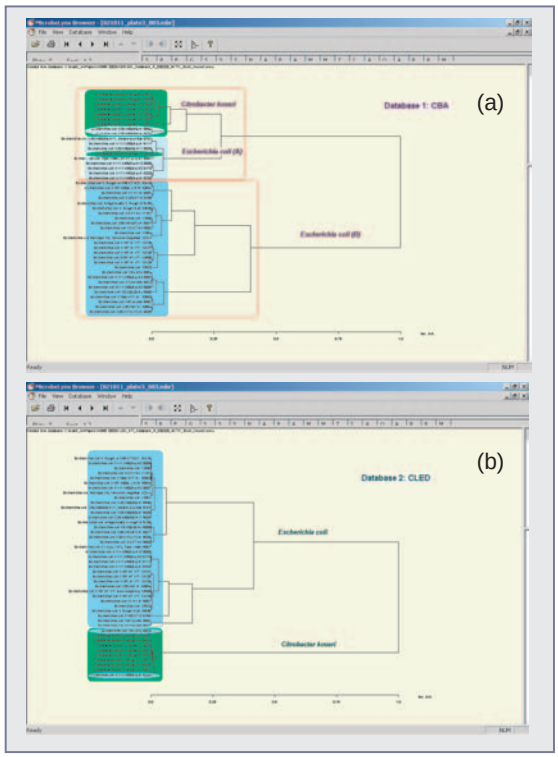

Figure 6. Dendograms produced from the cluster analysis of 37 strains of Escherichia coli & 8 strains of Citrobacter koseri for database 1: CBA & database 2: CLED respectively.

- Figure 6b also reveals 2 main clusters for the corresponding CLED database (database 2: CLED.)
  - The first contains 35 *Escherichia coli* species.
  - The second contains all 8 *Citrobacter koseri* species together with 2 aberrant Escherichia coli species NCTC 8333 & NCTC 9094.
  - *Escherichia coli* NCTC 8333 & NCTC 9094 spectra are very similar to those for *Citrobacter koseri* & lack the distinctive peaks at 2400 & 3850 Da.
  - The second cluster is much more homogeneous than the first.

3. The results of testing 20 clinical isolates of *Escherichia coli* against a combined database (CBA & CLED) containing a total of 1770 or 2159 spectral entries are given in Table 1.

No spectral entries			No. correctly identified Database 1: (CBA)	%	No. E. coli entries Database 1: (CBA)	No. correctly identified Database 2: (CLED)	%	No. E. coli entries Database: 2 (CLED)
CBA	CLED	Total						
1358	422	1770	7	35	54	15	75	37
1679	480	2159	7	35	54	15	75	37

Table 1. Results of testing 20 clinical isolates of Escherichia coli against combined databases containing 1770 & 2159 spectral entries.

- 35% correct identification is achieved using spectra generated using CBA media against 1770 or 2159 database entries.
- 75% correct identification is achieved using spectra generated using CLED agar against 1770 or 2159 database entries.
- Of the 5 isolates not identified using CLED agar:
  - 2 identified 6/8 & 5/8 matches as *Citrobacter koseri* & produced spectra similar to the aberrant *Escherichia coli* in Figure 5.
  - 1 gave top 6/8 matches as Morganella morganii.
  - 2 identified as Enterobacter cloacae.
- Spectra produced from cultures on CLED agar can differentiate between *Escherichia coli* & *Citrobacter koseri*.

CONCLUSION

1. The choice of culture media determines the MALDI-TOF mass spectral profile & has the potential to differentiate between biochemically similar species of *Escherichia coli* & *Citrobacter koseri*.
2. MALDI-TOF mass spectral profiles of *Escherichia coli* & *Citrobacter koseri* can generally be clustered according to species.
   - Greater discrimination can be achieved by changing the choice of media from CBA to CLED agar.
   - Cluster analysis can identify aberrant species requiring further investigation: *Escherichia coli* NCTC 8333 & 9094.

3&4. Successful identification of clinical isolate of *Escherichia coli* can be achieved within minutes using MALDI-TOF mass spectral analysis against a database of over 2000 spectral entries.

- 75% correct identification is achieved when clinical isolates are cultured using the more specialized CLED agar compared to 35% using CBA culture conditions.
- The results from the 5 misidentified isolates require further characterization using current "gold standard" techniques e.g. fatty acid methyl ester (FAME) analysis & 16S rDNA sequence analysis.

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Acknowledgements

The authors would like to thank Kerry Fleming, PHLS Chester, UK, for preparing the 20 clinical *Escherichia coli* isolates for analysis.

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