D. J. Dare¹, H. Sutton¹, X. Wang², H. Shah³, M. Lunt⁴; ¹Manchester Metropolitan University, Manchester, UK; ²New York City Department of Environmental Protection, Corona, NY; ³Health Protection Agency, London, UK; ⁴Waters Corporation, Manchester, UK

ABSTRACT

Waters

The City of New York Department of Environmental Protection, Beura water supply receives around 120 potable water samples/day for bacteriological analysis from the New York distribution system and reservoirs. Each sample is filtered though a membrane to isolate any bacterial contamination. These are then cultured, purified and identified by the VITEK system. The time taken for identification is 24 hours. To reduce the time taken for analysis MALDI-TOF mass spectrometry was investigated as a rapid method for identification.

Intact bacterial cells from individual colonies were transferred from a culture plate to a MALDI target well Each well was overlaid with the MALDI matrix alpha cyano-4-hydroxycinnamic acid. The co-crystallised sample was then irradiated with a N₂ laser and the resulting plume of positive ions separated using time-of-flight mass spectrometry. The characteristic fingerprint pattern produced, was identified against a database containing more than 3400 spectral entries (Figure 1). The time taken for analysis of each sample was of the order of 5 minutes.

A total of 14 samples were revived from slopes for MALDI-TOF MS analysis. Each sample was analysed twice, i) from cultures on CBA and ii) from cultures on CLED agar. The MALDI-TOF MS results were compared with the original VITEK identification. Eight of the 14 MALDI-TOF MS results from CBA agreed with the VITEK identification. One result was inconclusive. For the remaining 5 samples both techniques agree at the family level, but were different at lower levels of classification. One of the samples from CBA was identified to species with a high probability, the rest were split between two species from the same family. The results from the more specialised CLED agar demonstrated 12/14 results in agreement with the VITEK system. One result was inconclusive and the other matched a closely related species from the same family.

The results demonstrate that MALDI-TOF MS is a suitable rapid method for identification of bacteria from environmental water samples. Closely related *Enterobacteriaceae* are differentiated more easily using colonies cultured from the more exacting CLED medium.



Figure 1. Analysis of the characteristic mass spectral fingerprint pattern produced for sample 10 cultured on CLED agar against a database containing more than 3400 spectral entries.

METHODS

Environmental Test Isolates

Current method of identification

- Water samples (100 ml) collected from New York City's distribution sample sites in sterilized thio bottles with 0.1 ml of 10% sodium thiosulfate (Na₂S₂O₃).
- 100 ml of water sample was filtered through sterile 0.45 um nitrocellulose filter (Millipore, Bedford, Mass).
- The filter was placed in a 47 mm Petri dish containing 2 ml m-Endo broth, incubated at 35 +/- 0.5 °C for 22–24 hours.
- Any Red colonies with golden metallic sheen* were selected and transferred into Lauryl Typtose Broth (LTB), Brilliant green bile 2% (BGB), and EC with MUG (EC-methylumbelliferyl-B-Dglucuronide) media and incubated at 35 +/- 0.5 °C for 22–24 hours.
- If LTB and BGB produced gas and the colony morphology verified as Coliform, and if fermentation and fluorescence under UV (365 nm) was observed in EC/MUG then E. coli was confirmed.
- Positive LTB, BGB, and EC/MUG cultures were streaked onto MacConKey agar, incubated at 35 +/- 0.5 °C for 24 hours.
- Pink Colonies were picked and sub-cultured to Tryptic Soy Agar (TSA) incubated for a further 24 hours at 35 +/- 0.5 °C.

- The purified colonies on the TSA agar was used for MALDI-TOF mass spectrometry analysis and identified by the VITEK system.
- From the purified culture on TSA Gram stain and cytochrome oxidase tests were preformed. The culture was standardized and inoculated to the VITEK card, sealed and placed in the VITEK incubator, and identification results was read after 4 to 24 hours.

MALDI-TOF MS method of identification

- Seventeen environmental test isolates were selected/ purified as above by The City of New York Department of Environmental Protection, Beura water supply, and sent to MMU for analysis.
- The environmental water isolates were sub-cultured from Nutrient Agar slopes.
- Each isolate was inoculated onto two agar plates 1) Columbia Blood agar (CBA) plates containing 5% (v/v) horse blood (CBA: Oxoid, Basingstoke, UK.), incubated for 24 hours at 37 °C in an aerobic atmosphere;
 2) Cystine Lactose Electrolyte Deficient Agar (CLED) incubated for 16 hours at 35 °C in an aerobic atmosphere
- Two further sub cultures were made prior to MALDI-TOF analysis, thus ensuring that the isolate was fully recovered from shipment from the USA to the UK.
- All 3 subcultures were analysed blind and the results compared.

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; a-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 a-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

- All Database strains were cultured and analysed following strict protocols as described by Keys *et.al* 2004¹.
- A database was constructed which included ~3,400 spectral entries from NCTC strains covering >500 different species.
- 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 MicrobeLynxTM software.

- The average spectrum of each test strain was searched against the database of ~3,400 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 2.

RESULTS

- From the seventeen environmental test isolates received only 14 were viable.
- The VITEK and corresponding MALDI-TOF MS identification are shown in table 1 & 2, and demonstrate; Assuming the VITEK results are valid.



Figure 2. Browser results demonstrating the top 8 matches are all to Escherichia coli for Isolate 2 cultured from CLED agar, and analysed against a database of >3400 spectral entries. The spectral patterns for the top 2 matches are also presented.



Figure 3: Browser results demonstrating the top 8 matches are split between Salmonella enterica and Escherichia coli for Isolate 2 cultured from CBA agar and analysed against a database of >3400 spectral entries. The spectral patterns for the top 2 matches are also presented, demonstrating their similarity using this media.

- 8/14 Agree to Species.
- 5/14 Agree to Family.
- 1/14 Inconclusive MALDI-TOF MS Identification.

CLED

CBA

- 12/14 Agree to Species
- 1/14 Agree to Family.
- 1/14 Inconclusive MALDI-TOF MS Identification.



Figure 4. Browser results' demonstrating the 1st match is to Citrobacter freundii NCTC 6848, the remainder are generally to Escherichia coli for Isolate 8 cultured from CBA agar and analysed against a database of >3400 spectral entries. The spectral patterns for the top 2 matches are also presented, demonstrating their similarity using this media.



Figure 5. Browser results demonstrating the top 8 matches are all to Escherichia coli for Isolate 14 cultured from CLED agar, and analysed against a database of >3400 spectral entries. The spectral patterns for the top 2 matches are also presented.

Sample Numbers 2 and 5:

- VITEK identifies samples 2 and 5 as Escherichia coli.
- MALDI-TOF MS identification from CBA cultures were evenly split for the top 8 spectral matches, between 2 genera from the same family *Escherichia* (4/8) and *Salmonella* (4/8) (Figure 3).
- MALDI-TOF MS identification from CLED conclusively identified the samples as *Escherichia coli* (Figure 2).

Sample Numbers 8 and 14:

- VITEK identifies samples 8 and 14 as Escherichia coli.
- MALDI-TOF MS identification from CBA cultures for the top 8 spectral matches, is split between genera from the same family *Escherichia* (3/8) and *Citrobacter* (5/8) for sample 8. *Escherichia* (6/8), *Citrobacter* (1/8), *Salmonella* (1/8) for sample 14 (Figure 4).
- MALDI-TOF MS identification from CLED conclusively identified both samples as *Escherichia coli* (Figure 5).

Sample Number 7:

- VITEK identifies sample 8 as *Aeromonas sobria*. MALDI-TOF MS gave inconclusive results on both CBA and CLED
- Currently Aeromonas sobria is under represented in the MMU database, with only 4 NCTC strains represent this species. Earlier studies carried out indicate that the number of representative strains per species should be around 20 in order to increase the certainty of identification and cover the diversity within the species².

Sample Number 9:

- VITEK the identifies sample 9 as Klebsiella ozaenae. MALDI-TOF MS identified the sample consistently on both CBA and CLED as *Escherichia coli*.
- Currently *Klebsiella ozaenae* is under represented in the MMU database, with only 4 NCTC strains represent this species. Earlier studies carried out indicate that the number of representative strains per species should be around 20 in order to increase the certainty of identification and cover the diversity within the species².

CONCLUSION

- MALDI-TOF MS can be used to identify bacterial isolates from waters samples.
- MALDI-TOF MS is a rapid method for bacterial identification:
- Identification using MALDI-TOF MS requires minutes.
 Identification using the VITEK system require a 4 to 24
- hours.
- CLED medium provides enhanced discrimination of the Enterobacteriaceae Family.
- Two of the 14 samples failed to agree with the VITEK identification and require further analysis.

Sample Number	VITEK ID	MALDI ID (CBA)	Agreement
1	Serratia marcescens	Serratia marcescens	 ✓
2	Escherichia coli	Salmonella enterica	f
3	Escherichia coli	Escherichia coli	~
4	Escherichia coli	Escherichia coli	~
5	Escherichia coli	Salmonella enterica	f
6	Escherichia coli	Escherichia coli	 ✓
7	Aeromonas sobria	Inconclusive	Ş
8	Escherichia coli	Citrobacter freundii	f
9	Klebsiella ozaenae	Escherichia coli	f
10	Escherichia coli	Escherichia coli	~
11	Escherichia coli	Escherichia coli	 ✓
12	Escherichia coli	Escherichia coli	~
13	Escherichia coli	Escherichia coli	~
14	Escherichia coli	Citrobacter freundii	f

Table 1. CBA

Key: f = Same Family. Similar results were obtained for all 3 subcultures.

Sample Number	VITEK ID	Maldi ID (Cled)	Agreement
1	Serratia marcescens	Serratia marcescens	v
2	Escherichia coli	Escherichia coli	V
3	Escherichia coli	Escherichia coli	V
4	Escherichia coli	Escherichia coli	V
5	Escherichia coli	Escherichia coli	V
6	Escherichia coli	Escherichia coli	V
7	Aeromonas sobria	Inconclusive	Ś
8	Escherichia coli	Escherichia coli	V
9	Klebsiella ozaenae	Escherichia coli	f
10	Escherichia coli	Escherichia coli	V
11	Escherichia coli	Escherichia coli	~
12	Escherichia coli	Escherichia coli	v
13	Escherichia coli	Escherichia coli	V
14	Escherichia coli	Escherichia coli	~

Table 2. CLED

Key: f = Same Family. Similar results were obtained for all 3 subcultures.

Future work

- To further reduce time to results, the red colonies with golden metallic sheen could be transferred directly to the CLED/ CBA agar for analysis* from the initial m-Endo broth, thereby cutting down the number of culturing steps significantly.
- 2. Disparity between analysis of isolates should be investigated by a third type of analysis e.g. 16S rDNA or Fatty acid analysis.

References

- Keys C., Dare D., Sutton H., Wells G., Lunt M., McKenna T., McDowall M., Shah H., Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases, <u>Infection</u>, <u>Genetics and Evolution</u>; 2004, Volume 4; Issue 3, 221–242.
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