Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry: rapid identification of bacteria isolated from patients with cystic fibrosis

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ABSTRACT

Despite extensive research into the diagnosis and management of cystic fibrosis (CF) over the past decades, sufferers still have a median life expectancy of less than 37 years. Respiratory tract infections have a significant role in increasing the morbidity and mortality of patients with CF via a progressive decline in lung function. Rapid identification of organisms recovered from CF sputum is necessary for effective management of respiratory tract infections; however, standard techniques of identification are slow, technically demanding and expensive. The aim of this study is to asses the suitability of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) in identifying bacteria isolated from the respiratory tract of patients with CF, and is assessed by testing the accuracy of MALDI-TOF MS in identifying samples from a reference collection of rare CF strains in conjunction with comparing MALDI-TOF MS and standard techniques in identifying clinical isolates from sputum samples of CF patients. MALDI-TOF MS accurately identified 100% of isolates from the reference collection of rare CF pathogens (EuroCare CF collection). The isolate identification given by MALDI-TOF MS agreed with that given by standard techniques for 479/481 (99.6%) clinical isolates obtained from respiratory samples provided by patients with CF. In two (0.4%) of 481 samples there was a discrepancy in identification between MALDI-TOF MS and standard techniques. One organism was identified as *Pseudomonas aeruginosa* by MALDI-TOF but could only be identified by the laboratory's standard methods as of the *Pseudomonas* genus. The second organism was identified as *P. beteli* by MALDI-TOF MS and *Stenotrophomonas maltophilia* by standard methods. This study shows that MALDI-TOF MS is superior to standard techniques in providing cheap, rapid and accurate identification of CF sputum isolates.

KEY WORDS: Cystic fibrosis.
Microbiology.
Spectrometry, mass, matrix-assisted laser desorption-ionization.

Introduction

In patients affected by cystic fibrosis (CF), a genetic defect in the cystic fibrosis transmembrane conductance receptor (CFTR) transmembrane protein leads to altered airway fluid secretion, thickened mucus secretions and an increased susceptibility to severe recurrent respiratory infections.

While opportunistic *Pseudomonas aeruginosa* infection affects up to 80% of CF patients,[1] a number of other bacterial species have a significant role in the morbidity and mortality associated with CF. Research into the incidence of rarer infections has shown an overall increase in *Staphylococcus aureus* infections (including methicillin-resistant strains), an increase in *Stenotrophomonas maltophilia* and *Achromobacter* spp. infections and variations in infection incidence between members of the *Burkholderia cepacia* complex (BCC).[2]

Not all infections are equal in clinical significance. For instance, *Burkholderia cenocepacia* infection is associated with a significant increase in early mortality,[3] while certain strains of *P. aeruginosa* bring about greater annual decrease in lung function.[4] Clinical management and antimicrobial therapy vary depending on the infecting organism. Rapid and accurate identification of pathogens is crucial for early, appropriate treatment,[5,6] yet current methods of bacterial identification rely primarily on biochemical tests and have a limited ability to provide this.

The current process of identification in our laboratory is based on a diagnostic algorithm proposed by the UK CF Trust Microbiology Laboratory Standards Working Group,[5] and involves a combination of phenotypic and molecular techniques. These include commercial biochemical profiling kits such as the API 20 NE kit, growth on selective agar, polymyxin susceptibility, *P. aeruginosa*-specific exotoxin A polymerase chain reaction (PCR),[7,8] and 16S rDNA sequence analysis.

The reliability of phenotypic testing is greatly reduced in sputum samples from CF patients in comparison to those from unaffected individuals. This is due to the abnormal phenotype of the bacterial species. Altered conditions in the CF airway lead to changes in bacterial behaviour, including increase in biofilm formation and alteration in protein production. In addition, minor gene changes can affect molecular test results due to variations in the genes targeted by PCR amplification. As well as having limited accuracy, current methods are time consuming and, in the case of molecular testing, expensive and labour intensive.

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The recent introduction of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) to the field of microbiological identification has allowed rapid and accurate identification of bacteria using the specific mass spectral fingerprints of bacteria. The protein composition of bacteria is presented as mass spectra, and conserved or varying peaks can be used to group or separate bacteria, and MALDI-TOF MS has shown considerable potential in identifying clinical bacterial isolates.[9–11]

Degand *et al*. demonstrated that MALDI-TOF MS accurately identifies 98–100% of non-fermenting Gram-negative bacilli from CF patients when used in combination with a specifically created database.[12] In a separate study, Miñan *et al*. showed that MALDI-TOF MS was able to differentiate within the closely related species of the *B. cepacia* complex,[13] while Seng *et al*. envisioned MALDI TOF MS replacing biochemical testing as a method of clinical bacterial identification in the near future.[10]

In this study we aim to determine whether or not a commercially available MALDI-TOF MS system could identify accurately common CF pathogens (clinically isolated) and rare CF pathogens (from a reference collection).

Materials and methods

In order to determine if the MALDI-TOF system was capable of identifying more unusual strains of bacteria colonising CF patients, strains from the EuroCare CF collection[14] were analysed, as follows: *Achromobacter xylosoxidans* LMG 1863; *Burkholderia cenocepacia* LMG 12614, LMG 16654, LMG 16656, LMG 18829, LMG18830; *B. cepacia* LMG 1222; *B. dolosa* LMG 18943; *B. gladioli* LMG 18157; *B. multivorans* LMG 13010, LMG 16660, LMG 16775; *B. pyroccinia* LMG 21824; *B. stabilis* LMG 14294; *B. vietnamienis* LMG 18835; *Cupriavidus respiraculi* LMG 21510; *Inquilinus limosus* LMG 20952; *P. apista* LMG 16407; *P. pnomenusa* LMG 18087; *P. pulmonicola* LMG 1810; *Pandoarae sputorum* LMG 18819; *Ralstonia insidiosa* LMG 18101, *Ralstonia mannitolilytica* LMG 18103; *R. picketti* LMG 18088 and *S. maltophilia* LMG 958.

Additionally, clinical isolates (n=481) were recovered from the sputum samples of CF patients attending paediatric and adult CF clinics at Barts and the London NHS Trust. These patients had pulmonary exacerbations, were under surveillance for *Pseudomonas aeruginosa* acquisition or were known to be chronically colonised by CF-associated pathogens. No human samples were stored or tested, therefore ethical approval was not sought for this study.

All clinical isolates were initially identified by a range of methods in accordance with the laboratory's standard operating procedures. These included biochemical profiling such as phenotypic characteristics (Gram stain, growth on *Pseudomonas*- and *Burkholderia*-selective media), mucoidicity, oxidase test, biochemical profile obtained in the commercial biochemical kit API 20NE (bioMérieux, Basingstoke, UK), exotoxin A PCR and 16S rDNA sequence analysis. Samples were frozen at –70°C.

For MALDI-TOF analysis, the MALDI-TOF operator was blind to the initial identification of the isolates. The isolates were thawed, subcultured on Columbia blood agar and cultured at 37°C in air with 5% carbon dioxide for up to 72 h. A loop (1 µL) of a fresh bacterial colony was suspended in 300 µL water, vortex-mixed briefly and 900 µL 70% ethanol added. The suspension was centrifuged briefly at 10,000 x*g* for 3 min to deposit the bacterial pellet, and the supernatant removed and discarded. Peptides were extracted using 25 µL 70% formic acid and 25 µL acetonitrile.

The solution was transferred to the MALDI-TOF target plate and overlaid with 1 µL hydroxy cinnamic acid matrix (10 mg/mL). The target plates were analysed using the Bruker Microflex MALDI-TOF MS running Biotyper v2. The database used was Version 3.0.2.0. Results with a score <2.0 were discarded. Isolates giving results of 'no peaks found' (often a consequence of high protein concentration) were double diluted in acetonitrile (1 in 2, 1 in 4, 1 in 8) and rerun.

Results

All the EuroCare CF reference strains were correctly identified to species level (scores ≥2.2) by MALDI-TOF MS analysis without recourse to special methods of preparation or modification of the data base. This demonstrates the suitability of the commercial database for identification of isolates from CF patients.

The results obtained here by a combination of phenotypic, biochemical and molecular methods are referred to collectively as 'standard techniques' and are compared to those obtained by MALDI-TOF in Table 1. Nine of the isolates initially gave different identifications on MALDI-TOF MS analysis to those given by standard techniques, but seven of these concurred on repeat. These are listed in Table 2.

Discussion

Current methods of identifying pathogens causing CF respiratory tract infections are slow, costly and inaccurate. Rapid identification is key to the provision of effective and accurate treatment and management. Evidence shows that early and aggressive treatment of *P. aeruginosa* infection is

Table 1. Identification results in agreement for standard methods and MALDI-TOF MS.

No of isolates	Standard techniques	MALDI-TOF MS
413	*Pseudomonas aeruginosa*	*P. aeruginosa*
25	*Achromobacter xylosoxidans*	*A. xylosoxidans*
8	*Stenotrophomonas maltophilia*	*S. maltophilia*
8	*Pseudomonas monteilii*	*P. monteilii*
7	*Burkholderia cepacia* complex	*B. cepacia*
5	*Chryseobacterium indologenes*	*C. indologenes*
1	*Eschericihia coli*	*E. coli*
1	*Streptococcus australis*	*S. australis*
1	*Proteus mirabilis*	*P. mirabilis*
1	*Serratia marcescens*	*S. marcescens*
1	*Enterobacter cloacae*	*E. cloacae*
1	*Pseudomonas* sp.	*Pseudomonas libanesis*

Table 2. Identification results that differed between those obtained by standard techniques and by MALDI-TOF MS, and the results obtained on repeat testing.

No of isolates	Identification by standard techniques	Identification by MALDI-TOF MS	Repeat identification by standard technique	Repeat identification by MALDI-TOF MS
2	*S. maltophilia*	*P. beteli*	*S maltophilia*	*P. beteli*
1	*B. cepacia*	*A. xylosoxidans*	*A. xylosoxidans*	*A. xylosoxidans*
1	*R. pickettii*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*
3	*P. aeruginosa*	*A. xylosoxidans*	*A. xylosoxidans*	*A. xylosoxidans*
1	*P. aeruginosa*	*P. monteilii*	*P. monteilii*	*P. monteilii*
1	*Pseudomonas* sp.	*P. aeruginosa*	*Pseudomonas* sp.	*P. aeruginosa*

highly effective,[15] while delayed treatment leads to chronic infection that is often impossible to eradicate[9] and increases morbidity and mortality.[16] Patients infected with BCC are ideally given immediate, aggressive antibiotic therapy and are isolated from other CF patients to prevent spread.[5] If identification of isolates is delayed then these systems cannot be implemented effectively.

As well as misidentifying *P. aeruginosa* and *B. cepacia* species, current techniques also fail to detect numerous rarer species such as those included in the EuroCare CF collection. Only recent research with improved diagnostics can begin to describe the significance of these pathogens in CF pathology.

Furthermore, standard techniques also give a high rate of false positives. These have a serious detrimental effect on patient care due to unnecessary treatment, segregation on wards or clinics, and exclusion from lung transplant programmes (in the case of *B. cepacia* infection).[17]

In contrast to standard techniques, MALDI-TOF MS accurately identifies common pathogens such as *P. aeruginosa* and *B. cepacia* as well as the more unusual strains from the EuroCare CF collection. Similarly encouraging evidence of the ability of MALDI-TOF MS to recognise clinical isolates has been shown by Christner *et al*. in identifying blood culture samples,[13] by Miñan *et al*. in identifying bacteria of the novel taxon K class of BCC11 and by Fernandes Olmoz *et al*. in identifying unusual CF pathogens.[18]

One further advantage of the MALDI-TOF MS system is its ability to differentiate between bacteria to a more accurate level than current methods. A large proportion (38%) of the isolates could only be identified as 'non-fermentative Gram-negative bacilli (NFGNB) using standard techniques without the use of species-specific PCRs or 16S rDNA sequencing. This is mainly because the bacterial biochemical profile did not 'fit' with established species. Cystic Fibrosis Trust guidelines for best practice have highlighted that all NFGNB should be identified to species level as management and prognosis vary widely within the classification of NFGNB.[5] Here, MALDI-TOF MS provided immediate species identification for all 480 isolates.

Phenotypic tests were also unable to differentiate between species included in the BCC, such as *B. multivorans*, *B. dolosa* and *B. cepacia*, while MALDI TOF MS could. Although species within the BCC are closely related, it is important to differentiate between them as their presence has a variable effect on prognosis. *B. cenocepacia* in particular has been associated with increased morbidity in CF patients (more rapid reduction in FEV1 and weight loss) compared to other members of the BCC,[19] and colonisation with *B. cenocepacia* is an absolute contraindication to lung transplantation in many centres.

There are a number of explanations as to why MALDI-TOF MS provides an improved rate of identification in comparison to phenotypic tests. First, some bacteria, such as the BCC, *Ralstonia* species and *Pandoraea* species are rarely found in patients without CF,[2] and therefore they are not included in biochemical kit profiles. These organisms remain undetected or misidentified.

Second, the typical phenotypic behaviour used to identify common bacteria is altered, and thus response to variations in, for example, culture medium, growth conditions and antibiotics, give unpredictable and therefore inaccurate results.[20] Changes to bacterial behaviour and morphology are derived from quorum sensing, which is encouraged by the high bacterial cell density in the mucous secretions. Quorum sensing has been shown to affect pigment production,[21] mucoidicity and the formation of biofilms in *P. aeruginosa*,[22] which hinder current phenotypic identification techniques.

There were nine isolates in which the identification obtained by MALDI-TOF MS differed from that obtained by standard methods. These are shown in Table 2. All nine isolates were recultured from storage at –70°C and MALDI-TOF MS and standard identification tests were repeated. The results then concurred for seven of the nine results. The most likely explanation for the original difference in results is operator error in selecting the wrong isolate for freezing or mislabelling the cryovial after identification by standard methods.

The two isolates for which there remained a disagreement were labelled as *S. maltophilia* by standard methods and *P. beteli* by MALDI-TOF MS. A similar finding has been reported and discussed elsewhere.[23] Presently, it is not clear whether or not *S. maltophilia* and *P. beteli* are synonymous.

One isolate was identified as *P. aeruginosa* by MALDI-TOF MS but only as *Pseudomonas* sp. by standard methods in our laboratory, as it was negative for the *Pseudomonas aeruginosa* exotoxin A gene (ETA) by PCR. The ETA PCR assay relies on amplification of a fragment of the ETA. Disagreement over the amplicon size of the product has been described[16] and discrepancies between ETA PCR results and 16S PCR suggest that this method is not 100% specific or sensitive for *P. aeruginosa*[24,25] Mutation of the *eta* gene in CF strains is also probably promoted by the existence of hypermutators that readily emerge as the organism adapts to the environment of the CF lung.

Although MALDI-TOF MS has the ability to differentiate between species in the BCC, some closely related species were isolated that could not be distinguished by MALDI-TOF MS. As well as *P. beteli*, *P. hibiscicola*, *P. geniculata* and *S. maltophilia*, these include *A. xylosoxidans* and *A. ruhlandii*, *P. fulva* and *P. parafulva*, *P. monteili*, *P. putida* and *P. plecoglossicida*, and lastly *Streptococcus parasanguis* and *S. australis*. In the particular case of CF, treatment, management and prognosis do not vary between these groups of species; however, this highlights the inability of MALDI-TOF MS to differentiate between some closely related species that may be more significant in other cases.

Conclusions

The MALDI-TOF MS system provides an accurate, rapid, cheap and easy method of identification with the potential to improve the diagnosis and management of CF patients who have respiratory tract infections. We have not formally measured the reduction in turnaround time achieved using MALDI-TOF, as this depends on individual laboratory practice. However, 16S rRNA sequencing is performed in our laboratory twice a week and *P. aeruginosa* ETA PCR once a week; therefore, the identification of non-fermentative Gram-negative bacilli can often take up to seven days after isolation. In contrast, using MALDI-TOF, bacteria can be identified reliably on the day of isolation. Similar results were obtained by Degand *et al*.[12] and Fernández-Olmos *et al*.,[19] although, in the case of the former, a database was created for the identification of CF isolates and is not readily available to other users, severely limiting its usefulness. In this study, we used standard extraction methods and a commercially available database. Use of MALDI-TOF MS can provide more reliable and accurate results than current methods of diagnosis and can identify a much wider range of pathogens. Although the initial capital outlay is much larger, the running costs of MALDI-TOF are far lower than a combination of phenotypic and molecular tests, and reliable results are obtained much more quickly. □

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