A single nucleotide polymorphism identification assay for the genotypic characterisation of *Neisseria meningitidis* using MALDI-TOF mass spectrometry

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ABSTRACT

The ability of matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF) to identify virulent clones of meningococci quickly and accurately is investigated. A single nucleotide polymorphism (SNP) within the *fum*C gene which differentiates between the hypervirulent ET-15 strain and other ET-37 complex strains is used to determine the usefulness of this method. In this study, MALDI-TOF proved to be a fast, effective alternative to traditional DNA sequencing for the identification of an individual nucleotide.

KEY WORDS: Neisseria meningitidis.
Oligonucleotides.
Spectrometry, mass, matrix-assisted laser desorption-ionization.
Spectrum analysis, mass.

Introduction

Despite the introduction of meningococcal serogroup C polysaccharide conjugate (MenC) vaccines, *Neisseria meningitidis* remains an important disease with high rates of morbidity and mortality.[1,2] The introduction of MenC vaccines has led to a decrease in the incidence of serogroup C meningococcal disease in the UK[3] but the potential for other strains to occupy the niche left by this serogroup remains.[4,5] Therefore, there is a continued need for improved typing methods to provide rapid identification of emerging clones.

Although multilocus sequence typing (MLST) and other methods are now being used routinely in reference laboratories,[6,7] rapid techniques are required to provide data for public health management which may also be used in diagnostic laboratories. Matrix-assisted laser desorption-ionisation time-of-flight (MALDI-TOF) mass spectrometry is a rapid method for the accurate measurement of molecular mass,[8] and can be used to analyse short sections of DNA or individual nucleotides.

The aim of this study is to demonstrate that MALDI-TOF could be used as a rapid method for the detection of single nucleotide polymorphisms (SNPs) in certain meningococcal strains. In order to achieve this, the ET-15 G/A SNP at position 640 in the fumarate hydratase (*fumC*) gene is used to analyse clinical isolates from the ET-37 complex.[9]

Materials and methods

Ten ET-37 complex meningococcal strains were selected. The serotype and serosubtype of these organisms was determined previously by monoclonal antibody enzyme-linked immunosorbent assay (ELISA) or by nucleotide sequencing.[10,11] In addition, three ET15 isolates known to possess the *fumC* SNP were included.

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GCTTAAA**G**ACTTGTATGAACTTGCTTTGGGCG **1**
***TC**TGAACATACTTGAACGAAAC (6.6kDa)

GCTTAAA**A**ACTTGTATGAACTTGCTTTGGGCG **2**
***T**TGAACATACTTGAACGAAAC (6.4kDa)

Fig. 1. DNA sequence of the ET-15/37 single nucleotide polymorphism. Sequence of a section of the *fumC* gene containing the single nucleotide polymorphism (emboldened) and extension products of the wild-type ET-37 (1) and ET-15 (2) strains (***T** = dideoxythymidine terminating extension reaction).

Bacteria were stored, grown and their DNA extracted for polymerase chain reaction (PCR) analysis as described previously.[12] PCR amplification of the *fumC* gene[9] was performed on a Primus HT cyler (MWG-Biotech, Milton Keynes, UK). The PCR reaction was performed directly on 3 µL DNA extract using 20 µL ReddyMix PCR master mix (1 unit *Thermus aquaticus* [*Taq*] DNA polymerase, 1.3 mmol/L $MgCl_2$ and 0.2 mmol/L dNTPs; ABgene, Epsom, Surrey) and 25 pmoles each of forward (5′ CACCGAACACGACACGATGG) and reverse (5′-ACGACCAGTTCGTCAAACTC) primers (MWG-Biotech). The total PCR reaction volume was 25 µL.

An initial denaturisation step of 95°C was followed by 45 cycles of 95°C for 1 min, 54.5°C for 1 min 30 sec, and 72°C for 2 min. The reaction was completed with a final 5 min

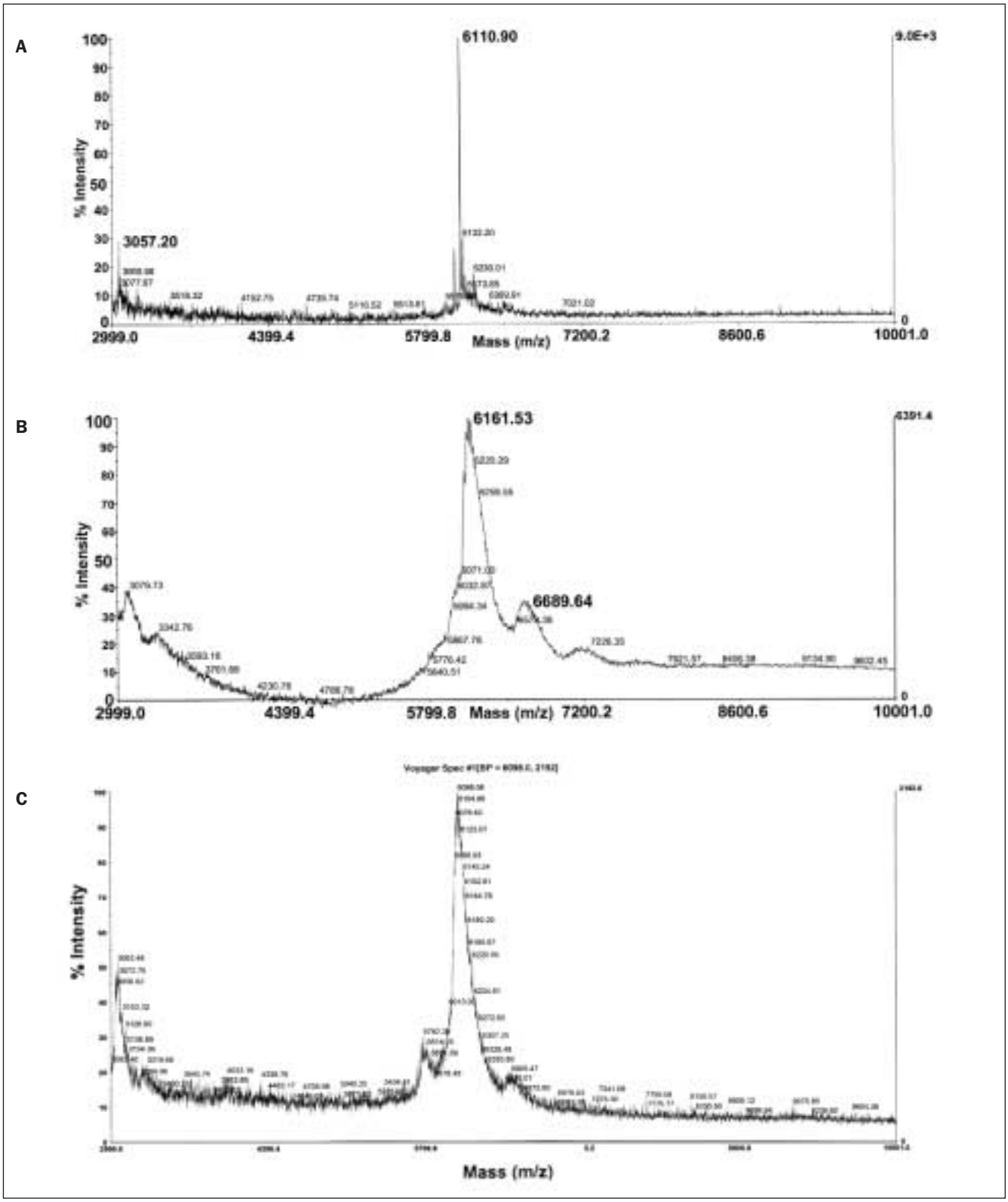

Fig. 2. Mass spectrometry results from a) primer extension, b) G allele extension and c) A allele extension.

at 72°C. After cycling, the PCR fragment size of 1.3 kb was confirmed by agarose gel (1.5%) electrophoresis using 5 µL of the sample. The PCR product was cleaned using a Multiscreen PCR clean-up plate (Millipore, Watford, UK), as described previously.[13]

Oligonucleotides for MALDI-TOF analysis were created by primer extension using the primer 5′-CAAAGCAAGTTCATACAAGT (MWG-Biotech). The primer extension reaction consisted of 1 unit *Taq* DNA polymerase in the *Taq* buffer supplied (Amersham Biosciences, Little Chalfont, UK), 2.5 mmol/L $MgCl_2$, 60 pmoles primer (MWG-Biotech), 0.1 mmol/L ddTTP (Amersham Biosciences), 0.1 mmol/L dCTP (Amersham Biosciences) and 0.5 µL purified PCR product in a final

volume of 5 µL. The reaction mix was denatured at 95˚C for 2 minutes, followed by 30 cycles of 95˚C for 15 seconds, 48˚C for 30 seconds and 70˚C for 30 seconds, followed by a final extension of 72˚C for 1 min.

For MALDI-TOF analysis, a 2 µL sample was taken directly from the primer extension reaction and diluted in 3 µL matrix (50 mg 3-hydroxypicolinic acid in a mixture of 750 mL 30% acetonitrile and 250 µL 0.2 mol/L ammonium citrate, creating a saturated solution). Using this mixture, a series of two 1:1 (2 µL into 2µl of fresh matrix) dilutions were performed.

Approximately 1.7 µL sample/matrix mix was spotted onto a metal plate and air-dried to allow crystal formation. The two dilutions were examined by MALDI-TOF, as was the pure extension primer that was used initially to set up the mass spectrometer to read oligonucleotides.

A Voyager-DE Pro Biospectrometry Workstation mass spectrometer (Applied Biosystems, Warrington, UK) was used, set in linear negative mode. The accelerating voltage was at 18,000 V with a grid of 95%, guide wire of 0.2% and laser intensity of approximately 80-85%. The time delay between each laser shot was set at 900 nsec and 200 shots were used to compile each spectrum.

Results

From each of the 10 meningococci identified phenotypically as ET-37 meningococcal strains, a section of the *fumC* gene containing the SNP was amplified by PCR. The oligonucleotide primer annealed immediately upstream of the point mutation site and a primer extension carried out in the presence of only two nucleotides (dCTP and a terminator dideoxythymidine triphosphate [ddTTP]).

The DNA sequence of the SNP and the adjacent nucleotide determined that the length of the extension can only be one or two bases, depending upon the identity of the SNP (Fig. 1). The additional molecular weight added to the primer was determined by MALDI-TOF and used to identify the DNA sequence of the oligonucleotide.

Three distinguishable peaks of different molecular weights were observed after MALDI-TOF analysis. These were of approximately 6.2 kDa, 6.4 kDa and 6.6k Da (Fig. 2). From their nucleotide composition it was estimated that the primer alone would be 6.118 kDa, the G allele extension product 6.695 kDa and the A allele 6.406 kDa. All samples tested were found to produce a secondary peak of the size expected.

Using the protocol described it was possible to screen the meningococcal strains quickly, which illustrated the potential usefulness of this technique when rapid results are required, as is the case during a case cluster.

Discussion

The use of MALDI-TOF to screen clinical isolates containing a known SNP offers a faster and more cost-effective alternative to traditional nucleotide sequencing. A direct mass measurement is available within a few minutes, with no need for nucleotide sequence data analysis. The method also offers the possibility of easy conversion to automation.

In this study, the resulting G or A allele oligonucleotides did not require an additional purification step to achieve sufficient resolution to make them distinguishable from the original primer because a high primer concentration was used. However, an improvement in the efficiency of the primer extension reaction would aid the identification of the second peak. Simple methods of improving the resolution of the sample that would not add significantly to preparation times, such as changes to the composition of the matrix, need to be investigated.

In summary, the technique described could be used as a rapid method to detect SNPs in certain meningococcal strains and give valuable epidemiological information on the emergence and spread of these strains. □

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